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(54) Title: USE OF ANTAGONIST ANTI-CD40 ANTIBODIES FOR TREATMENT OF CHRONIC LYMPHOCYTIC LEUKEMIA

(57) Abstract: Methods of therapy for treating a subject for chronic lymphocytic leukemia are provided. The methods comprise administering a therapeutically effective amount of an antagonist anti-CD40 antibody or antigen-binding fragment thereof to a patient in need thereof. The antagonist anti-CD40 antibody or antigen-binding fragment thereof is free of significant agonist activity, but exhibits antagonist activity when the antibody binds a CD40 antigen on a human CD40-expressing cell. Antagonist activity of the anti-CD40 antibody or antigen-binding fragment thereof beneficially inhibits proliferation and/or differentiation of human CD40-expressing chronic lymphocytic leukemia cells.

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USE OF ANTAGONIST ANTI-CD40 MONOCLONAL ANTIBODIES FOR TREATMENT OF CHRONIC LYMPHOCYTIC LEUKEMIA

FIELD OF THE INVENTION

The invention relates to methods for treatment of chronic lymphocytic leukemia using antagonist anti-CD40 monoclonal antibodies.

5

BACKGROUND OF THE INVENTION

Chronic lymphocytic leukemia (CLL) is a B cell malignancy characterized by neoplastic cell proliferation and accumulation in bone marrow, blood, lymph nodes, and the spleen. CLL is the most common type of adult leukemia in the Western 10 hemisphere. Incidence of CLL increases in the aging population, with the median age at time of diagnosis being about 65 years. Current treatment protocols include chemotherapeutic agents such as fludarabine, 2-chlorodeoxyadenosine (cladribine), chlorambucil, vincristine, pentostatin, cyclophosphamide, alemtuzumab (Campath-1H), doxorubicin, and prednisone. Fludarabine is the most effective 15 chemotherapeutic with response rates of 17 to 74%, but CLL often becomes refractory to repeated courses of the drug (Rozman and Montserrat (1995) *NEJM* 2133:1052).

The median survival rate for CLL patients is nine years, although some 20 patients with mutated immunoglobulin genes have a more favorable prognosis. See, for example, Rozman and Montserrat (1995) *NEJM* 2133:1052) and Keating *et al.* (2003) *Hematol.* 2003:153. In cases where CLL has transformed into large-cell lymphoma, median survival drops to less than one year; similarly, cases of 25 prolymphocytic leukemia have a poorer prognosis than classical CLL (Rozman and Montserrat (1995) *NEJM* 2133:1052). To date, no evidence for a cure has been obtained.

CD40 is a 55 kDa cell-surface antigen present on the surface of both normal and neoplastic human B cells, dendritic cells, other antigen presenting cells (APCs), endothelial cells, monocytic cells, and epithelial cells. Binding of the CD40 ligand to the CD40 antigen on the B cell membrane provides a positive costimulatory signal
5 that stimulates B cell activation and proliferation, resulting in B cell maturation into a plasma cell that secretes high levels of soluble immunoglobulin. Transformed cells from patients with low- and high-grade B-cell lymphomas, B-cell acute lymphoblastic leukemia, CLL, myeloblastic leukemia, and Hodgkin's disease express CD40.
Malignant B cells from several tumors of B-cell lineage express a high level of CD40
10 and appear to depend on CD40 signaling for survival and proliferation. This renders the CD40 antigen a potential target for anti-cancer therapy.

Given the poor prognosis for patients with chronic lymphocytic leukemia, alternative treatment protocols are needed.

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BRIEF SUMMARY OF THE INVENTION

Methods are provided for treating a human subject with chronic lymphocytic leukemia (CLL), comprising administering to the subject an anti-CD40 antibody or an antigen-binding fragment thereof that is free of significant agonist activity when bound to a CD40 antigen on a human CD40-expressing cell. Methods for inhibiting
20 growth of CLL cells expressing CD40 antigen are also provided.

Suitable antagonist anti-CD40 antibodies for use in the methods of the present invention have a strong affinity for CD40 and are characterized by a dissociation equilibrium constant (K_D) of at least 10^{-6} M, preferably at least about 10^{-7} M to about 10^{-8} M, more preferably at least about 10^{-8} M to about 10^{-12} M. These monoclonal
25 antibodies and antigen-binding fragments thereof are capable of specifically binding to human CD40 antigen expressed on the surface of a human cell. They are free of significant agonist activity but exhibit antagonist activity when bound to CD40 antigen on human cells. In one embodiment, the anti-CD40 antibody or fragment thereof exhibits antagonist activity when bound to CD40 antigen on normal human B cells. In another embodiment, the anti-CD40 antibody or fragment thereof exhibits antagonist activity when bound to CD40 antigen on malignant human B cells.
30 Suitable monoclonal antibodies have human constant regions; preferably they also

have wholly or partially humanized framework regions; and most preferably are fully human antibodies or antigen-binding fragments thereof. Examples of such monoclonal antibodies are the antibodies designated herein as CHIR-5.9 and CHIR-12.12, which can be recombinantly produced; the monoclonal antibodies produced by 5 the hybridoma cell lines designated 131.2F8.5.9 (referred to herein as the cell line 5.9) and 153.8E2.D10.D6.12.12 (referred to herein as the cell line 12.12); a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:6, the sequence shown in SEQ ID NO:7, the sequence shown in SEQ ID NO:8, both the sequence shown in SEQ ID NO:6 and SEQ ID 10 NO:7, and both the sequence shown in SEQ ID NO:6 and SEQ ID NO:8; a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:2, the sequence shown in SEQ ID NO:4, the sequence shown in SEQ ID NO:5, both the sequence shown in SEQ ID NO:2 and SEQ ID NO:4, and both the sequence shown in SEQ ID NO:2 and SEQ ID 15 NO:5; a monoclonal antibody comprising an amino acid sequence encoded by a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of the sequence shown in SEQ ID NO:1, the sequence shown in SEQ ID NO:3, and both the sequence shown in SEQ ID NO:1 and SEQ ID NO:3; and antigen-binding fragments of these monoclonal antibodies that retain the capability of 20 specifically binding to human CD40, and which are free of significant agonist activity but exhibit antagonist activity when bound to CD40 antigen on human cells.

Examples of such monoclonal antibodies also include a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12; a monoclonal antibody that binds to an epitope 25 comprising residues 82-87 of the amino acid sequence shown in SEQ ID NO:10 or SEQ ID NO:12; a monoclonal antibody that competes with the monoclonal antibody CHIR-5.9 or CHIR-12.12 in a competitive binding assay; and a monoclonal antibody that is an antigen-binding fragment of the CHIR-5.9 or CHIR-12.12 monoclonal antibody or any of the foregoing monoclonal antibodies, where the fragment retains 30 the capability of specifically binding to the human CD40 antigen.

In one embodiment of the invention, methods of treatment comprise administering to a patient a therapeutically effective dose of a pharmaceutical

composition comprising suitable antagonistic anti-CD40 antibodies or antigen-binding fragments thereof. A therapeutically effective dose of the anti-CD40 antibody or fragment thereof is in the range from about 0.01 mg/kg to about 40 mg/kg, from about 0.01 mg/kg to about 30 mg/kg, from about 0.1 mg/kg to about 30 mg/kg, from about 1 mg/kg to about 30 mg/kg, from about 3 mg/kg to about 30 mg/kg, from about 3 mg/kg to about 25 mg/kg, from about 3 mg/kg to about 20 mg/kg, from about 5 mg/kg to about 15 mg/kg, or from about 7 mg/kg to about 12 mg/kg. It is recognized that the method of treatment may comprise a single administration of a therapeutically effective dose or multiple administrations of a therapeutically effective dose of the antagonist anti-CD40 antibody or antigen-binding fragment thereof.

The antagonist anti-CD40 antibodies identified herein as being suitable for use in the methods of the invention may be modified. Modifications of these antagonist anti-CD40 antibodies include, but are not limited to, immunologically active chimeric anti-CD40 antibodies, humanized anti-CD40 antibodies, and immunologically active murine anti-CD40 antibodies.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 sets forth the amino acid sequences for the light and heavy chains of the mAb CHIR-12.12. The leader (residues 1-20 of SEQ ID NO:2), variable (residues 21-132 of SEQ ID NO:2), and constant (residues 133-239 of SEQ ID NO:2) regions of the light chain are shown in Figure 1A. The leader (residues 1-19 of SEQ ID NO:4), variable (residues 20-139 of SEQ ID NO:4), and constant (residues 140-469 of SEQ ID NO:4) regions of the heavy chain are shown in Figure 1B. The alternative constant region for the heavy chain of the mAb CHIR-12.12 shown in Figure 1B reflects a substitution of a serine residue for the alanine residue at position 153 of SEQ ID NO:4. The complete sequence for this variant of the heavy chain of the mAb CHIR-12.12 is set forth in SEQ ID NO:5.

Figure 2 shows the coding sequence for the light chain (Figure 2A; SEQ ID NO:1) and heavy chain (Figure 2B; SEQ ID NO:3) for the mAb CHIR-12.12.

Figure 3 sets forth the amino acid sequences for the light and heavy chains of mAb CHIR-5.9. The leader (residues 1-20 of SEQ ID NO:6), variable (residues 21-

132 of SEQ ID NO:6), and constant (residues 133-239 of SEQ ID NO:6) regions of the light chain are shown in Figure 3A. The leader (residues 1-19 of SEQ ID NO:7), variable (residues 20-144 of SEQ ID NO:7), and constant (residues 145-474 of SEQ ID NO:7) regions of the heavy chain are shown in Figure 3B. The alternative 5 constant region for the heavy chain of the mAb CHIR-5.9 shown in Figure 3B reflects a substitution of a serine residue for the alanine residue at position 158 of SEQ ID NO:7. The complete sequence for this variant of the heavy chain of the mAb CHIR-5.9 is set forth in SEQ ID NO:8.

Figure 4 shows the coding sequence (Figure 4A; SEQ ID NO:9) for the short 10 isoform of human CD40 (amino acid sequence shown in Figure 4B; SEQ ID NO:10), and the coding sequence (Figure 4C; SEQ ID NO:11) for the long isoform of human CD40 (amino acid sequence shown in Figure 4D).

Figure 5 shows that monoclonal antibody CHIR-12.12 inhibits CD40L-mediated proliferation of cancer cells from patients with CLL (n=9) at 48h (Figure 15 5A) and 72h (Figure 5B).

Figure 6 shows that monoclonal antibody CHIR-12.12 does not have a stimulatory effect on CLL patient cells (n=9) at 48h (Figure 6A) and 72h (Figure 6B).

Figure 7 shows more efficient ADCC-mediated cell lysis of CLL cell line EHEB by monoclonal antibody CHIR-12.12 versus the monoclonal antibody 20 Rituxan®.

Figure 8 shows thermal melting temperature of CHIR-12.12 in different pH formulations measured by differential scanning calorimetry (DSC).

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DETAILED DESCRIPTION OF THE INVENTION

"Tumor," as used herein, refers to all neoplastic cell growth and proliferation, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. 30 Examples of cancer include but are not limited to lymphomas and leukemias.

"Antibodies" and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to an antigen, immunoglobulins include both antibodies and other antibody-like molecules that lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

5 The term "antibody" is used in the broadest sense and covers fully assembled antibodies, antibody fragments that can bind antigen (e.g., Fab', F'(ab)₂, Fv, single chain antibodies, diabodies), and recombinant peptides comprising the foregoing.

The term "monoclonal antibody" as used herein refers to an antibody obtained 10 from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts.

"Native antibodies" and "native immunoglobulins" are usually 15 heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. 20 Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light- and heavy-chain variable domains.

25 The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) 30 or hypervariable regions both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are celled the framework (FR) regions. The variable domains of native heavy and light chains each comprise

four FR regions, largely adopting a β -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β -sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat *et al.* (1991) *NIH Publ. No. 91-3242*, Vol. I, pages 647-669).

The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions, such as Fc receptor (FcR) binding, participation of the antibody in antibody-dependent cellular toxicity, opsonization, initiation of complement dependent cytotoxicity, and mast cell degranulation.

The term "hypervariable region" when used herein refers to the amino acid residues of an antibody that are responsible for antigen binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (i.e., residues 24-34 (L1), 50-56 (L2), and 89-97 (L3) in the light-chain variable domain and 31-35 (H1), 50-65 (H2), and 95-102 (H3) in the heavy-chain variable domain; Kabat *et al.* (1991) *Sequences of Proteins of Immunological Interest* (5th ed., Public Health Service, National Institute of Health, Bethesda, MD) and/or those residues from a "hypervariable loop" (i.e., residues 26-32(L1), 50-52 (L2), and 91-96 (L3) in the light-chain variable domain and 26-32(H1), 53-55 (H2), and 96-101 (H3) in the heavy-chain variable domain; Clothia and Lesk (1987) *J. Mol. Biol.* 196:901-917). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues.

"Antibody fragments" comprise a portion of an intact antibody, preferably the antigen-binding or variable region of the intact antibody. Examples of antibody fragments include Fab, Fab', F(ab')2, and Fv fragments; diabodies; linear antibodies (Zapata *et al.* (1995) *Protein Eng.* 8(10):1057-1062); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an F(ab')2 fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

"Fv" is the minimum antibody fragment that contains a complete antigen recognition and binding site. In a two-chain Fv species, this region consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv species, one heavy- and one light-chain variable domain can be 5 covalently linked by flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable 10 domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment also contains the constant domain of the light chain and the first constant domain (C_H1) of the heavy chain. Fab fragments differ from Fab' 15 fragments by the addition of a few residues at the carboxy terminus of the heavy-chain C_H1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')2 antibody fragments originally were produced as pairs of Fab' fragments that have hinge cysteines between them. Other 20 chemical couplings of antibody fragments are also known.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequence of the constant domain of their heavy 25 chains, immunoglobulins can be assigned to different classes. There are five major classes of human immunoglobulins: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2. The heavy-chain constant domains that correspond to the different classes of immunoglobulins are called alpha, delta, epsilon, gamma, and mu, respectively. 30 The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known. Different isotypes have different effector

functions. For example, human IgG1 and IgG3 isotypes mediate antibody-dependent cell-mediated cytotoxicity (ADCC) activity.

The word "label" when used herein refers to a detectable compound or composition that is conjugated directly or indirectly to the antibody so as to generate a 5 "labeled" antibody. The label may be detectable by itself (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may catalyze chemical alteration of a substrate compound or composition that is detectable. Radionuclides that can serve as detectable labels include, for example, I-131, I-123, I-125, Y-90, Re-188, Re-186, At-211, Cu-67, Bi-212, and Pd-109. The label might also be a non-10 detectable entity such as a toxin.

The term "antagonist" is used in the broadest sense, and includes any molecule that partially or fully blocks, inhibits, or neutralizes a biological activity of a native target disclosed herein or the transcription or translation thereof.

"Carriers" as used herein include pharmaceutically acceptable carriers, 15 excipients, or stabilizers that are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, succinate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than 20 about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN, 25 polyethylene glycol (PEG), and Pluronics. Administration "in combination with" one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

A "host cell," as used herein, refers to a microorganism or a eukaryotic cell or 30 cell line cultured as a unicellular entity that can be, or has been, used as a recipient for a recombinant vector or other transfer polynucleotides, and include the progeny of the original cell that has been transfected. It is understood that the progeny of a single

cell may not necessarily be completely identical in morphology or in genomic or total DNA complement as the original parent, due to natural, accidental, or deliberate mutation.

"Human effector cells" are leukocytes that express one or more FcRs and 5 perform effector functions. Preferably, the cells express at least Fc γ RIII and carry out antigen-dependent cell-mediated cytotoxicity (ADCC) effector function. Examples of human leukocytes that mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, macrophages, eosinophils, and neutrophils, with PBMCs and NK cells being preferred. Antibodies that have ADCC 10 activity are typically of the IgG1 or IgG3 isotype. Note that in addition to isolating IgG1 and IgG3 antibodies, such ADCC-mediating antibodies can be made by engineering a variable region from a non-ADCC antibody or variable region fragment to an IgG1 or IgG3 isotype constant region.

The terms "Fc receptor" or "FcR" are used to describe a receptor that binds to 15 the Fc region of an antibody. The preferred FcR is a native-sequence human FcR. Moreover, a preferred FcR is one that binds an IgG antibody (a gamma receptor) and includes receptors of the Fc γ RI, Fc γ RII, and Fc γ RIII subclasses, including allelic variants and alternatively spliced forms of these receptors. Fc γ RII receptors include Fc γ RIIA (an "activating receptor") and Fc γ RIIB (an "inhibiting receptor"), which 20 have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor Fc γ RIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor Fc γ RIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain (see Daeron (1997) *Annu. Rev. Immunol.* 15:203-234). FcRs are reviewed in 25 Ravetch and Kinet (1991) *Annu. Rev. Immunol.* 9:457-492 (1991); Capel *et al.* (1994) *Immunomethods* 4:25-34; and de Haas *et al.* (1995) *J. Lab. Clin. Med.* 126:330-341. Other FcRs, including those to be identified in the future, are encompassed by the 30 term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al.* (1976) *J. Immunol.* 117:587 and Kim *et al.* (1994) *J. Immunol.* 24:249 (1994)).

There are a number of ways to make human antibodies. For example, secreting cells can be immortalized by infection with the Epstein-Barr virus (EBV).

However, EBV-infected cells are difficult to clone and usually produce only relatively low yields of immunoglobulin (James and Bell (1987) *J. Immunol. Methods* 100:5-40). In the future, the immortalization of human B cells might possibly be achieved by introducing a defined combination of transforming genes. Such a possibility is highlighted by a recent demonstration that the expression of the telomerase catalytic subunit together with the SV40 large oncprotein and an oncogenic allele of H-ras resulted in the tumorigenic conversion of normal human epithelial and fibroblast cells (Hahn *et al.* (1999) *Nature* 400:464-468). It is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a repertoire of human antibodies in the absence of endogenous immunoglobulin production (Jakobovits *et al.* (1993) *Nature* 362:255-258; Lonberg and Huszar (1995) *Int. Rev. Immunol.* 13:65-93; Fishwild *et al.* (1996) *Nat. Biotechnol.* 14:845-851; Mendez *et al.* (1997) *Nat. Genet.* 15:146-156; Green (1999) *J. Immunol. Methods* 231:11-23; Tomizuka *et al.* (2000) *Proc. Natl. Acad. Sci. USA* 97:722-727; reviewed in Little *et al.* (2000) *Immunol. Today* 21:364-370). For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production (Jakobovits *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90:2551-2555). Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice results in the production of human antibodies upon antigen challenge (Jakobovits *et al.* (1993) *Nature* 362:255-258). Mendez *et al.* (1997) (*Nature Genetics* 15:146-156) have generated a line of transgenic mice that, when challenged with an antigen, generates high affinity fully human antibodies. This was achieved by germ-line integration of megabase human heavy-chain and light-chain loci into mice with deletion into endogenous J_H segment as described above. These mice (XenoMouse® II technology (Abgenix; Fremont, California)) harbor 1,020 kb of human heavy-chain locus containing approximately 66 V_H genes, complete D_H and J_H regions, and three different constant regions, and also harbors 800 kb of human κ locus containing 32 $V\kappa$ genes, $J\kappa$ segments, and $C\kappa$ genes. The antibodies produced in these mice closely resemble that seen in humans in all respects, including gene rearrangement, assembly, and repertoire. The human antibodies are preferentially expressed over endogenous

antibodies due to deletion in endogenous segment that prevents gene rearrangement in the murine locus. Such mice may be immunized with an antigen of particular interest.

Sera from such immunized animals may be screened for antibody reactivity against the initial antigen. Lymphocytes may be isolated from lymph nodes or spleen 5 cells and may further be selected for B cells by selecting for CD138-negative and CD19-positive cells. In one aspect, such B cell cultures (BCCs) may be fused to myeloma cells to generate hybridomas as detailed above.

In another aspect, such B cell cultures may be screened further for reactivity 10 against the initial antigen, preferably. Such screening includes ELISA with the target/antigen protein, a competition assay with known antibodies that bind the antigen of interest, and in vitro binding to transiently transfected CHO or other cells that express the target antigen.

The present invention is directed to compositions and methods for treating 15 human subjects having chronic lymphocytic leukemia (CLL). The methods involve treatment with an anti-CD40 antibody described herein, or an antigen-binding fragment thereof, where administration of the antibody or antigen-binding fragment thereof promotes a positive therapeutic response within the subject undergoing this method of therapy. Anti-CD40 antibodies suitable for use in the methods of the invention specifically bind a human CD40 antigen expressed on the surface of a 20 human cell and are free of significant agonist activity, but exhibit antagonist activity when bound to the CD40 antigen on a human CD40-expressing cell, as demonstrated for CD40-expressing normal and neoplastic human B cells, including CLL cells. These anti-CD40 antibodies and antigen-binding fragments thereof are referred to herein as "antagonist anti-CD40 antibodies." Such antibodies include, but are not 25 limited to, the fully human monoclonal antibodies CHIR-5.9 and CHIR-12.12 described below and monoclonal antibodies having the binding characteristics of monoclonal antibodies CHIR-5.9 and CHIR-12.12, also described below. These monoclonal antibodies, which can be recombinantly produced, are discussed below and disclosed in the copending provisional applications entitled "*Antagonist Anti-* 30 *CD40 Monoclonal Antibodies and Methods for Their Use,*" filed November 4, 2003, November 26, 2003, and April 27, 2004, and assigned U.S. Patent Application Nos. 60/517,337 (Attorney Docket No. PP20107.001 (035784/258442)), 60/525,579

(Attorney Docket No. PP20107.002 (035784/271525)), and 60/565,710 (Attorney Docket No. PP20107.003 (035784/277214)), respectively, the contents of each of which are herein incorporated by reference in their entirety.

Antibodies that have the binding characteristics of monoclonal antibodies
5 CHIR-5.9 and CHIR-12.12 include antibodies that competitively interfere with binding CD40 and/or bind the same epitopes as CHIR-5.9 and CHIR-12.12. One of skill in the art could determine whether an antibody competitively interferes with CHIR-5.9 or CHIR-12.12 using standard methods known in the art.

When these antibodies bind CD40 displayed on the surface of human cells,
10 such as human B cells, the antibodies are free of significant agonist activity; in some embodiments, their binding to CD40 displayed on the surface of human cells results in inhibition of proliferation and differentiation of these human cells. Thus, the antagonist anti-CD40 antibodies suitable for use in the methods of the invention include those monoclonal antibodies that can exhibit antagonist activity toward
15 normal and malignant human cells expressing the cell-surface CD40 antigen.

Antagonist Anti-CD40 Antibodies

The monoclonal antibodies CHIR-5.9 and CHIR-12.12 represent suitable antagonist anti-CD40 antibodies for use in the methods of the present invention. The
20 CHIR-5.9 and CHIR-12.12 antibodies are fully human anti-CD40 monoclonal antibodies of the IgG₁ isotype produced from the hybridoma cell lines 131.2F8.5.9 (referred to herein as the cell line 5.9) and 153.8E2.D10.D6.12.12 (referred to herein as the cell line 12.12). These cell lines were created using splenocytes from immunized xenotypic mice containing the human IgG₁ heavy chain locus and the
25 human κ chain locus (XenoMouse® technology; Abgenix; Fremont, California). The spleen cells were fused with the mouse myeloma SP2/0 cells (Sierra BioSource). The resulting hybridomas were sub-cloned several times to create the stable monoclonal cell lines 5.9 and 12.12. Other antibodies of the invention may be prepared similarly using mice transgenic for human immunoglobulin loci or by other methods known in
30 the art and/or described herein.

The nucleotide and amino acid sequences of the variable regions of the CHIR-12.12 antibody, and the amino acid sequences of the variable regions of the CHIR-5.9

antibody, are disclosed in copending provisional applications entitled "*Antagonist Anti-CD40 Monoclonal Antibodies and Methods for Their Use*," filed November 4, 2003, November 26, 2003, and April 27, 2004, and assigned U.S. Patent Application Nos. 60/517,337 (Attorney Docket No. PP20107.001 (035784/258442)), 60/525,579 5 (Attorney Docket No. PP20107.002 (035784/271525)), and 60/565,710 (Attorney Docket No. PP20107.003 (035784/277214)), respectively, the contents of each of which are herein incorporated by reference in their entirety. The amino acid sequences for the leader, variable, and constant regions for the light chain and heavy chain for mAb CHIR-12.12 are set forth herein in Figures 1A and 1B, respectively.

10 See also SEQ ID NO:2 (complete sequence for the light chain of mAb CHIR-12.12), SEQ ID NO:4 (complete sequence for the heavy chain for mAb CHIR-12.12), and SEQ ID NO:5 (complete sequence for a variant of the heavy chain for mAb CHIR-12.12 set forth in SEQ ID NO:4, where the variant comprises a serine substitution for the alanine residue at position 153 of SEQ ID NO:4). The nucleotide sequences 15 encoding the light chain and heavy chain for mAb CHIR-12.12 are set forth herein in Figures 2A and 2B, respectively. See also SEQ ID NO:1 (coding sequence for the light chain for mAb CHIR-12.12), and SEQ ID NO:3 (coding sequence for the heavy chain for mAb CHIR-12.12). The amino acid sequences for the leader, variable, and constant regions for the light chain and heavy chain of the CHIR-5.9 mAb are set 20 forth herein in Figures 3A and 3B, respectively. See also SEQ ID NO:6 (complete sequence for the light chain of mAb CHIR-5.9), SEQ ID NO:7 (complete sequence for the heavy chain of mAb CHIR-5.9), and SEQ ID NO:8 (complete sequence for a variant of the heavy chain of mAb CHIR-5.9 set forth in SEQ ID NO:7, where the variant comprises a serine substitution for the alanine residue at position 158 of SEQ 25 ID NO:7). Further, hybridomas expressing CHIR-5.9 and CHIR-12.12 antibodies have been deposited with the ATCC with a patent deposit designation of PTA-5542 and PTA-5543, respectively.

In addition to antagonist activity, it is preferable that anti-CD40 antibodies of this invention have another mechanism of action against a tumor cell. For example, 30 native CHIR-5.9 and CHIR-12.12 antibodies have ADCC activity. Alternatively, the variable regions of the CHIR-5.9 and CHIR-12.12 antibodies can be expressed on another antibody isotype that has ADCC activity. It is also possible to conjugate

native forms, recombinant forms, or antigen-binding fragments of CHIR-5.9 or CHIR-12.12 to a cytotoxin, a therapeutic agent, or a radioactive metal ion or radioisotope, as noted herein below.

The CHIR-5.9 and CHIR-12.12 monoclonal antibodies bind soluble CD40 in
5 ELISA-type assays, prevent the binding of CD40-ligand to cell-surface CD40, and
displace the pre-bound CD40-ligand, as determined by flow cytometric assays.
Antibodies CHIR-5.9 and CHIR-12.12 compete with each other for binding to CD40
but not with 15B8, the anti-CD40 monoclonal antibody described in U.S. Provisional
Application Serial No. 60/237,556, titled "*Human Anti-CD40 Antibodies*," filed
10 October 2, 2000, and PCT International Application No. PCT/US01/30857, also
titled "*Human Anti-CD40 Antibodies*," filed October 2, 2001 (Attorney Docket No.
PP16092.003), both of which are herein incorporated by reference in their entirety.
When tested *in vitro* for effects on proliferation of B cells from normal human
subjects, CHIR-5.9 and CHIR-12.12 act as antagonistic anti-CD40 antibodies.
15 Furthermore, CHIR-5.9 and CHIR-12.12 do not induce strong proliferation of human
lymphocytes from normal subjects. These antibodies are able to kill CD40-expressing
target cells by antibody dependent cellular cytotoxicity (ADCC). The binding affinity
of CHIR-5.9 for human CD40 is 1.2×10^{-8} M and the binding affinity of CHIR-12.12 is
 5×10^{-10} M, as determined by the BiacoreTM assay.
20 Suitable antagonist anti-CD40 antibodies for use in the methods of the present
invention exhibit a strong single-site binding affinity for the CD40 cell-surface
antigen. The monoclonal antibodies of the invention exhibit a dissociation
equilibrium constant (K_D) for CD40 of at least 10^{-5} M, at least 3×10^{-5} M, preferably
at least 10^{-6} M to 10^{-7} M, more preferably at least 10^{-8} M to about 10^{-12} M, measured
25 using a standard assay such as BiacoreTM. Biacore analysis is known in the art and
details are provided in the "BIAapplications handbook." Methods described in WO
01/27160 can be used to modulate the binding affinity.

By "CD40 antigen," "CD40 cell surface antigen," "CD40 receptor," or "CD40"
is intended a transmembrane glycoprotein that belongs to the tumor necrosis factor
30 (TNF) receptor family (see, for example, U.S. Patent Nos. 5,674,492 and 4,708,871;
Stamenkovic *et al.* (1989) *EMBO* 8:1403; Clark (1990) *Tissue Antigens* 36:33;
Barclay *et al.* (1997) *The Leucocyte Antigen Facts Book* (2d ed.; Academic Press, San

Diego)). Two isoforms of human CD40, encoded by alternatively spliced transcript variants of this gene, have been identified. The first isoform (also known as the "long isoform" or "isoform 1") is expressed as a 277-amino-acid precursor polypeptide (SEQ ID NO:12 (first reported as GenBank Accession No. CAA43045, and identified 5 as isoform 1 in GenBank Accession No. NP_001241), encoded by SEQ ID NO:11 (see GenBank Accession Nos. X60592 and NM_001250)), which has a signal sequence represented by the first 19 residues. The second isoform (also known as the "short isoform" or "isoform 2") is expressed as a 203-amino-acid precursor polypeptide (SEQ ID NO:10 (GenBank Accession No. NP_690593), encoded by SEQ 10 ID NO:9 (GenBank Accession No. NM_152854)), which also has a signal sequence represented by the first 19 residues. The precursor polypeptides of these two isoforms of human CD40 share in common their first 165 residues (i.e., residues 1-165 of SEQ ID NO:10 and SEQ ID NO:12). The precursor polypeptide of the short isoform (shown in SEQ ID NO:10) is encoded by a transcript variant (SEQ ID NO:9) that 15 lacks a coding segment, which leads to a translation frame shift; the resulting CD40 isoform contains a shorter and distinct C-terminus (residues 166-203 of SEQ ID NO:10) from that contained in the long isoform of CD40 (C-terminus shown in residues 166-277 of SEQ ID NO:12). For purposes of the present invention, the term "CD40 antigen," "CD40 cell surface antigen," "CD40 receptor," or "CD40" 20 encompasses both the short and long isoforms of CD40. The anti-CD40 antibodies of the present invention bind to an epitope of human CD40 that resides at the same location within either the short isoform or long isoform of this cell surface antigen as noted herein below.

The CD40 antigen is displayed on the surface of a variety of cell types, as 25 described elsewhere herein. By "displayed on the surface" and "expressed on the surface" is intended that all or a portion of the CD40 antigen is exposed to the exterior of the cell. The displayed or expressed CD40 antigen may be fully or partially glycosylated.

By "agonist activity" is intended that the substance functions as an agonist. 30 An agonist combines with a receptor on a cell and initiates a reaction or activity that is similar to or the same as that initiated by the receptor's natural ligand. For example, an agonist of CD40 induces any or all of, but not limited to, the following responses:

B cell proliferation and differentiation, antibody production, intercellular adhesion, B cell memory generation, isotype switching, up-regulation of cell-surface expression of MHC Class II and CD80/86, and secretion of pro-inflammatory cytokines such as IL-8, IL-12, and TNF. By "antagonist activity" is intended that the substance functions 5 as an antagonist. For example, an antagonist of CD40 prevents or reduces induction of any of the responses induced by binding of the CD40 receptor to an agonist ligand, particularly CD40L. The antagonist may reduce induction of any one or more of the responses to agonist binding by 5%, 10%, 15%, 20%, 25%, 30%, 35%, preferably 40%, 45%, 50%, 55%, 60%, more preferably 70%, 80%, 85%, and most preferably 10 90%, 95%, 99%, or 100%. Methods for measuring anti-CD40 antibody and CD40-ligand binding specificity and antagonist activity are known to one of skill in the art and include, but are not limited to, standard competitive binding assays, assays for monitoring immunoglobulin secretion by B cells, B cell proliferation assays, Banchereau-Like-B cell proliferation assays, T cell helper assays for antibody 15 production, co-stimulation of B cell proliferation assays, and assays for up-regulation of B cell activation markers. See, for example, such assays disclosed in WO 00/75348, U.S. Patent No. 6,087,329, and copending provisional applications entitled "Antagonist Anti-CD40 Monoclonal Antibodies and Methods for Their Use," filed November 4, 2003, November 26, 2003, and April 27, 2004, and assigned U.S. Patent 20 Application Nos. 60/517,337 (Attorney Docket No. PP20107.001 (035784/258442)), 60/525,579 (Attorney Docket No. PP20107.002 (035784/271525)), and 60/565,710 (Attorney Docket No. PP20107.003 (035784/277214)), respectively; the contents of each of which are herein incorporated by reference in their entirety.

By "significant" agonist activity is intended an agonist activity of at least 30%, 25 35%, 40%, 45%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% greater than the agonist activity induced by a neutral substance or negative control as measured in an assay of a B cell response. Preferably, "significant" agonist activity is an agonist activity that is at least 2-fold greater or at least 3-fold greater than the agonist activity induced by a neutral substance or negative control as measured in an assay of a B cell 30 response. Thus, for example, where the B cell response of interest is B cell proliferation, "significant" agonist activity would be induction of a level of B cell proliferation that is at least 2-fold greater or at least 3-fold greater than the level of B

cell proliferation induced by a neutral substance or negative control. In one embodiment, a non-specific immunoglobulin, for example IgG1, that does not bind to CD40 serves as the negative control. A substance "free of significant agonist activity" would exhibit an agonist activity of not more than about 25% greater than the agonist 5 activity induced by a neutral substance or negative control, preferably not more than about 20% greater, 15% greater, 10% greater, 5% greater, 1% greater, 0.5% greater, or even not more than about 0.1% greater than the agonist activity induced by a neutral substance or negative control as measured in an assay of a B cell response. The antagonist anti-CD40 antibodies useful in the methods of the present invention 10 are free of significant agonist activity as noted above when bound to a CD40 antigen on a human cell. In one embodiment of the invention, the antagonist anti-CD40 antibody is free of significant agonist activity in one B cell response. In another embodiment of the invention, the antagonist anti-CD40 antibody is free of significant agonist activity in assays of more than one B cell response (e.g., proliferation and 15 differentiation, or proliferation, differentiation, and antibody production).

As used herein "anti-CD40 antibody" encompasses any antibody that specifically recognizes the CD40 B cell surface antigen, including polyclonal antibodies, monoclonal antibodies, single-chain antibodies, and fragments thereof such as Fab, F(ab')₂, F_v, and other fragments which retain the antigen binding function 20 of the parent anti-CD40 antibody. Of particular interest to the methods of the present invention are antagonist anti-CD40 antibodies that share the binding characteristics of the monoclonal antibodies CHIR-5.9 and CHIR-12.12 described above.

Thus, in addition to the monoclonal antibodies CHIR-5.9 and CHIR-12.12, other antibodies that would be useful in practicing the methods of the invention 25 described herein include, but are not limited to, the following: (1) the monoclonal antibodies produced by the hybridoma cell lines designated 131.2F8.5.9 (referred to herein as the cell line 5.9) and 153.8E2.D10.D6.12.12 (referred to herein as the cell line 12.12), deposited with the ATCC as Patent Deposit No. PTA-5542 and Patent Deposit No. PTA-5543, respectively; (2) a monoclonal antibody comprising an amino 30 acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:2, the sequence shown in SEQ ID NO:4, the sequence shown in SEQ ID NO:5, both the sequences shown in SEQ ID NO:2 and SEQ ID NO:4, and both the

sequences shown in SEQ ID NO:2 and SEQ ID NO:5; (3) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:6, the sequence shown in SEQ ID NO:7, the sequence shown in SEQ ID NO:8, both the sequences shown in SEQ ID NO:6 and SEQ ID NO:7, and both the sequences shown in SEQ ID NO:6 and SEQ ID NO:8; (4) a monoclonal antibody having an amino acid sequence encoded by a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of the nucleotide sequence shown in SEQ ID NO:1, the nucleotide sequence shown in SEQ ID NO:3, and both the sequences shown in SEQ ID NO:1 and SEQ ID NO:3; (5) a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 5.9 or the hybridoma cell line 12.12; (6) a monoclonal antibody that binds to an epitope comprising residues 82-87 of the amino acid sequence shown in SEQ ID NO:10 or SEQ ID NO:12; (7) a monoclonal antibody that competes with the monoclonal antibody CHIR-5.9 or CHIR-12.12 in a competitive binding assay; and (8) a monoclonal antibody that is an antigen-binding fragment of the CHIR-12.12 or CHIR-5.9 monoclonal antibody or the foregoing monoclonal antibodies in preceding items (1)-(7), where the fragment retains the capability of specifically binding to the human CD40 antigen. Those skilled in the art recognize that the antagonist anti-CD40 antibodies and antigen-binding fragments of these antibodies suitable for use in the methods disclosed herein include antagonist anti-CD40 antibodies and antigen-binding fragments thereof that are produced recombinantly using methods well known in the art and described herein below, and include, for example, monoclonal antibodies CHIR-5.9 and CHIR-12.12 that have been recombinantly produced.

25

Production of Anti-CD40 Antibodies

The antagonist anti-CD40 antibodies for use in the methods of the present invention can be produced using any of the methods well known to those of skill in the art. Polyclonal sera may be prepared by conventional methods. In general, a solution containing the CD40 antigen is first used to immunize a suitable animal, preferably a mouse, rat, rabbit, or goat. Rabbits or goats are preferred for the

preparation of polyclonal sera due to the volume of serum obtainable, and the availability of labeled anti-rabbit and anti-goat antibodies.

Polyclonal sera can be prepared in a transgenic animal, preferably a mouse bearing human immunoglobulin loci. In a preferred embodiment, Sf9 cells expressing 5 CD40 are used as the immunogen. Immunization can also be performed by mixing or emulsifying the antigen-containing solution in saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion parenterally (generally subcutaneously or intramuscularly). A dose of 50-200 µg/injection is typically sufficient. Immunization is generally boosted 2-6 weeks later with one or 10 more injections of the protein in saline, preferably using Freund's incomplete adjuvant. One may alternatively generate antibodies by *in vitro* immunization using methods known in the art, which for the purposes of this invention is considered equivalent to *in vivo* immunization. Polyclonal antisera are obtained by bleeding the immunized animal into a glass or plastic container, incubating the blood at 25°C for 15 one hour, followed by incubating at 4°C for 2-18 hours. The serum is recovered by centrifugation (e.g., 1,000 x g for 10 minutes). About 20-50 ml per bleed may be obtained from rabbits.

Production of the Sf 9 (*Spodoptera frugiperda*) cells is disclosed in U.S. Patent No. 6,004,552, incorporated herein by reference. Briefly, sequences encoding 20 human CD40 were recombined into a baculovirus using transfer vectors. The plasmids were co-transfected with wild-type baculovirus DNA into Sf 9 cells. Recombinant baculovirus- infected Sf 9 cells were identified and clonally purified.

Preferably the antibody is monoclonal in nature. By "monoclonal antibody" is intended an antibody obtained from a population of substantially homogeneous 25 antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. The term is not limited regarding the species or source of the antibody. The term encompasses whole immunoglobulins as well as fragments such as Fab, F(ab')2, Fv, and others which retain the antigen binding function of the antibody. Monoclonal antibodies are highly specific, being directed against a single antigenic site, i.e., the 30 CD40 cell surface antigen in the present invention. Furthermore, in contrast to conventional (polyclonal) antibody preparations that typically include different

antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al.* (1975) *Nature* 256:495, or may be made by recombinant DNA methods (see, e.g., U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in, for example, Clackson *et al.* (1991) *Nature* 352:624-628; Marks *et al.* (1991) *J. Mol. Biol.* 222:581-597; and U.S. Patent No. 5,514,548.

By "epitope" is intended the part of an antigenic molecule to which an antibody is produced and to which the antibody will bind. Epitopes can comprise linear amino acid residues (i.e., residues within the epitope are arranged sequentially one after another in a linear fashion), nonlinear amino acid residues (referred to herein as "nonlinear epitopes"; these epitopes are not arranged sequentially), or both linear and nonlinear amino acid residues.

The term "CD40-antigen epitope" as used herein refers to a three dimensional molecular structure (either linear or conformational) that is capable of immunoreactivity with the anti-CD40 monoclonal antibodies of this invention, excluding the CD40 antigen itself. CD40-antigen epitopes may comprise proteins, protein fragments, peptides, carbohydrates, lipids, and other molecules, but for the purposes of the present invention are most commonly proteins, short oligopeptides, oligopeptide mimics (i.e., organic compounds which mimic the antibody binding properties of the CD40 antigen), or combinations thereof. Suitable oligopeptide mimics are described, inter alia, in PCT application US 91/04282.

Monoclonal antibodies can be prepared using the method of Kohler *et al.* (1975) *Nature* 256:495-496, or a modification thereof. Typically, a mouse is immunized with a solution containing an antigen. Immunization can be performed by mixing or emulsifying the antigen-containing solution in saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion

parenterally. Any method of immunization known in the art may be used to obtain the monoclonal antibodies of the invention. After immunization of the animal, the spleen (and optionally, several large lymph nodes) are removed and dissociated into single cells. The spleen cells may be screened by applying a cell suspension to a plate 5 or well coated with the antigen of interest. The B cells expressing membrane bound immunoglobulin specific for the antigen bind to the plate and are not rinsed away. Resulting B cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybridomas, and are cultured in a selective medium. The resulting cells are plated by serial dilution and are assayed for the production of 10 antibodies that specifically bind the antigen of interest (and that do not bind to unrelated antigens). The selected monoclonal antibody (mAb)-secreting hybridomas are then cultured either *in vitro* (e.g., in tissue culture bottles or hollow fiber reactors), or *in vivo* (as ascites in mice).

As an alternative to the use of hybridomas, antibody can be produced in a cell 15 line such as a CHO cell line, as disclosed in U.S. Patent Nos. 5,545,403; 5,545,405; and 5,998,144; incorporated herein by reference. Briefly the cell line is transfected with vectors capable of expressing a light chain and a heavy chain, respectively. By transfecting the two proteins on separate vectors, chimeric antibodies can be produced. Another advantage is the correct glycosylation of the antibody.

Monoclonal antibodies to CD40 are known in the art. See, for example, the 20 sections dedicated to B-cell antigen in McMichael, ed. (1987; 1989) *Leukocyte Typing III and IV* (Oxford University Press, New York); U.S. Patent Nos. 5,674,492; 5,874,082; 5,677,165; 6,056,959; WO 00/63395; International Publication Nos. WO 02/28905 and WO 02/28904; Gordon *et al.* (1988) *J. Immunol.* 140:1425; Valle *et al.* 25 (1989) *Eur. J. Immunol.* 19:1463; Clark *et al.* (1986) *PNAS* 83:4494; Paulie *et al.* (1989) *J. Immunol.* 142:590; Gordon *et al.* (1987) *Eur. J. Immunol.* 17:1535; Jabara *et al.* (1990) *J. Exp. Med.* 172:1861; Zhang *et al.* (1991) *J. Immunol.* 146:1836; Gascan *et al.* (1991) *J. Immunol.* 147:8; Banchereau *et al.* (1991) *Clin. Immunol. Spectrum* 3:8; and Banchereau *et al.* (1991) *Science* 251:70; all of which are herein incorporated 30 by reference. Of particular interest to the present invention are the antagonist anti-CD40 antibodies disclosed herein that share the binding characteristics of the monoclonal antibodies CHIR-5.9 and CHIR-12.12 described above.

Additionally, the term "anti-CD40 antibody" as used herein encompasses chimeric anti-CD40 antibodies; such chimeric anti-CD40 antibodies for use in the methods of the invention have the binding characteristics of the CHIR-5.9 and CHIR-12.12 monoclonal antibodies described herein. By "chimeric" antibodies is intended 5 antibodies that are most preferably derived using recombinant deoxyribonucleic acid techniques and which comprise both human (including immunologically "related" species, e.g., chimpanzee) and non-human components. Rituxan® is an example of a chimeric antibody with a murine variable region and a human constant region. For purposes of the present invention, the constant region of the chimeric antibody is most 10 preferably substantially identical to the constant region of a natural human antibody; the variable region of the chimeric antibody is most preferably derived from a non-human source and has the desired antigenic specificity to the CD40 cell-surface antigen. The non-human source can be any vertebrate source that can be used to generate antibodies to a human CD40 cell-surface antigen or material comprising a 15 human CD40 cell-surface antigen. Such non-human sources include, but are not limited to, rodents (e.g., rabbit, rat, mouse, etc.; see, for example, U.S. Patent No. 4,816,567, herein incorporated by reference) and non-human primates (e.g., Old World Monkey, Ape, etc.; see, for example, U.S. Patent Nos. 5,750,105 and 5,756,096; herein incorporated by reference). As used herein, the phrase 20 "immunologically active" when used in reference to chimeric anti-CD40 antibodies means a chimeric antibody that binds human CD40.

Humanized anti-CD40 antibodies represent additional anti-CD40 antibodies suitable for use in the methods of the present invention. By "humanized" is intended forms of anti-CD40 antibodies that contain minimal sequence derived from non- 25 human immunoglobulin sequences. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region (also known as complementarity determining region or CDR) of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit, or nonhuman primate having the desired 30 specificity, affinity, and capacity. The phrase "complementarity determining region" refers to amino acid sequences which together define the binding affinity and specificity of the natural Fv region of a native immunoglobulin binding site. See, e.g.,

Chothia *et al* (1987) *J. Mol. Biol.* 196:901-917; Kabat *et al* (1991) U. S. Dept. of Health and Human Services, NIH Publication No. 91-3242). The phrase "constant region" refers to the portion of the antibody molecule that confers effector functions. In previous work directed towards producing non-immunogenic antibodies for use in therapy of human disease, mouse constant regions were substituted by human constant regions. The constant regions of the subject humanized antibodies were derived from human immunoglobulins. However, these humanized antibodies still elicited an unwanted and potentially dangerous immune response in humans and there was a loss of affinity. Humanized anti-CD40 antibodies for use in the methods of the present invention have binding characteristics similar to those exhibited by the CHIR-5.9 and CHIR-12.12 monoclonal antibodies described herein.

Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al*. (1986) *Nature* 321:522-525; Riechmann *et al*. (1988) *Nature* 332:323-327; Verhoeyen *et al*. (1988) *Science* 239:1534-1536), by substituting rodent or mutant rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. See also U.S. Patent Nos. 5,225,539; 5,585,089; 5,693,761; 5,693,762; 5,859,205; herein incorporated by reference. In some instances, residues within the framework regions of one or more variable regions of the human immunoglobulin are replaced by corresponding non-human residues (see, for example, U.S. Patent Nos. 5,585,089; 5,693,761; 5,693,762; and 6,180,370). Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance (e.g., to obtain desired affinity). In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the framework regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details see Jones *et al*. (1986) *Nature* 331:522-525; Riechmann *et al*. (1988) *Nature* 332:323-329; and Presta (1992) *Curr. Op. Struct. Biol.* 2:593-596; herein incorporated by reference. Accordingly, such "humanized" antibodies may include antibodies wherein

substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some framework residues are substituted by residues from analogous sites in rodent 5 antibodies. See, for example, U.S. Patent Nos. 5,225,539; 5,585,089; 5,693,761; 5,693,762; 5,859,205. See also U.S. Patent No. 6,180,370, and International Publication No. WO 01/27160, where humanized antibodies and techniques for producing humanized antibodies having improved affinity for a predetermined antigen are disclosed.

10 Also encompassed by the term anti-CD40 antibodies are xenogeneic or modified anti-CD40 antibodies produced in a non-human mammalian host, more particularly a transgenic mouse, characterized by inactivated endogenous immunoglobulin (Ig) loci. In such transgenic animals, competent endogenous genes for the expression of light and heavy subunits of host immunoglobulins are rendered 15 non-functional and substituted with the analogous human immunoglobulin loci. These transgenic animals produce human antibodies in the substantial absence of light or heavy host immunoglobulin subunits. See, for example, U.S. Patent Nos. 5,877,397 and 5,939,598, herein incorporated by reference.

20 Preferably, fully human antibodies to CD40 are obtained by immunizing transgenic mice. One such mouse is obtained using XenoMouse® technology (Abgenix; Fremont, California), and is disclosed in U.S. Patent Nos. 6,075,181, 6,091,001, and 6,114,598, all of which are incorporated herein by reference. To produce the antibodies disclosed herein, mice transgenic for the human Ig G₁ heavy chain locus and the human K light chain locus were immunized with Sf 9 cells 25 expressing human CD40. Mice can also be transgenic for other isotypes. Fully human antibodies useful in the methods of the present invention are characterized by binding properties similar to those exhibited by the CHIR-5.9 and CHIR-12.12 monoclonal antibodies disclosed herein.

30 Fragments of the anti-CD40 antibodies are suitable for use in the methods of the invention so long as they retain the desired affinity of the full-length antibody. Thus, a fragment of an anti-CD40 antibody will retain the ability to bind to the CD40 B cell surface antigen. Such fragments are characterized by properties similar to the

corresponding full-length antagonist anti-CD40 antibody, that is, the fragments will specifically bind a human CD40 antigen expressed on the surface of a human cell, and are free of significant agonist activity but exhibit antagonist activity when bound to a CD40 antigen on a human CD40-expressing cell. Such fragments are referred to
5 herein as "antigen-binding" fragments.

Suitable antigen-binding fragments of an antibody comprise a portion of a full-length antibody, generally the antigen-binding or variable region thereof. Examples of antibody fragments include, but are not limited to, Fab, F(ab')₂, and Fv fragments and single-chain antibody molecules. By "Fab" is intended a monovalent
10 antigen-binding fragment of an immunoglobulin that is composed of the light chain and part of the heavy chain. By F(ab')₂ is intended a bivalent antigen-binding fragment of an immunoglobulin that contains both light chains and part of both heavy chains. By "single-chain Fv" or "sFv" antibody fragments is intended fragments comprising the V_H and V_L domains of an antibody, wherein these domains are present
15 in a single polypeptide chain. See, for example, U.S. Patent Nos. 4,946,778, 5,260,203, 5,455,030, and 5,856,456, herein incorporated by reference. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains that enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun (1994) in *The Pharmacology of Monoclonal Antibodies*,
20 Vol. 113, ed. Rosenburg and Moore (Springer-Verlag, New York), pp. 269-315.
Antigen-binding fragments of the antagonist anti-CD40 antibodies disclosed herein can also be conjugated to a cytotoxin to effect killing of the target cancer cells, as described herein below.

Antibodies or antibody fragments can be isolated from antibody phage
25 libraries generated using the techniques described in, for example, McCafferty *et al.* (1990) *Nature* 348:552-554 (1990) and U.S. Patent No. 5,514,548. Clackson *et al.* (1991) *Nature* 352:624-628 and Marks *et al.* (1991) *J. Mol. Biol.* 222:581-597 describe the isolation of murine and human antibodies, respectively, using phage
libraries. Subsequent publications describe the production of high affinity (nM range)
30 human antibodies by chain shuffling (Marks *et al.* (1992) *Bio/Technology* 10:779-783), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.* (1993) *Nucleic Acids Res.*

21:2265-2266). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived *via* proteolytic digestion of intact antibodies (see, e.g., Morimoto *et al.* (1992) *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al.* (1985) *Science* 229:81). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form F(ab')₂ fragments (Carter *et al.* (1992) *Bio/Technology* 10:163-167). According to another approach, F(ab')₂ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

Antagonist anti-CD40 antibodies useful in the methods of the present invention include the CHIR-5.9 and CHIR-12.12 monoclonal antibodies disclosed herein as well as antibodies differing from this antibody but retaining the CDRs; and antibodies with one or more amino acid addition(s), deletion(s), or substitution(s), wherein the antagonist activity is measured by inhibition of B-cell proliferation and/or differentiation. The invention also encompasses de-immunized antagonist anti-CD40 antibodies, which can be produced as described in, for example, International Publication Nos. WO 98/52976 and WO 0034317; herein incorporated by reference. In this manner, residues within the antagonist anti-CD40 antibodies of the invention are modified so as to render the antibodies non- or less immunogenic to humans while retaining their antagonist activity toward human CD40-expressing cells, wherein such activity is measured by assays noted elsewhere herein. Also included within the scope of the claims are fusion proteins comprising an antagonist anti-CD40 antibody of the invention, or a fragment thereof, which fusion proteins can be synthesized or expressed from corresponding polynucleotide vectors, as is known in the art. Such fusion proteins are described with reference to conjugation of antibodies as noted below.

The antibodies of the present invention can have sequence variations produced using methods described in, for example, Patent Publication Nos. EP 0 983 303 A1,

WO 00/34317, and WO 98/52976, incorporated herein by reference. For example, it has been shown that sequences within the CDR can cause an antibody to bind to MHC Class II and trigger an unwanted helper T-cell response. A conservative substitution can allow the antibody to retain binding activity yet lose its ability to 5 trigger an unwanted T-cell response. Any such conservative or non-conservative substitutions can be made using art-recognized methods, such as those noted elsewhere herein, and the resulting antibodies will fall within the scope of the invention. The variant antibodies can be routinely tested for antagonist activity, affinity, and specificity using methods described herein.

10 An antibody produced by any of the methods described above, or any other method not disclosed herein, will fall within the scope of the invention if it possesses at least one of the following biological activities: inhibition of immunoglobulin secretion by normal human peripheral B cells stimulated by T cells; inhibition of proliferation of normal human peripheral B cells stimulated by Jurkat T cells; 15 inhibition of proliferation of normal human peripheral B cells stimulated by CD40L-expressing cells or soluble CD40; and inhibition of proliferation of human malignant B cells as noted below. These assays can be performed as described in copending provisional applications entitled "*Antagonist Anti-CD40 Monoclonal Antibodies and Methods for Their Use*," filed November 4, 2003, November 26, 2003, and April 27, 20 2004, and assigned U.S. Patent Application Nos. 60/517,337 (Attorney Docket No. PP20107.001 (035784/258442)), 60/525,579 (Attorney Docket No. PP20107.002 (035784/271525)), and 60/565,710 (Attorney Docket No. PP20107.003 (035784/277214)), respectively; the contents of each of which are herein incorporated by reference in their entirety. See also the assays described in Schultze *et al.* (1998) 25 *Proc. Natl. Acad. Sci. USA* 92:8200-8204; Denton *et al.* (1998) *Pediatr. Transplant.* 2:6-15; Evans *et al.* (2000) *J. Immunol.* 164:688-697; Noelle (1998) *Agents Actions Suppl.* 49:17-22; Lederman *et al.* (1996) *Curr. Opin. Hematol.* 3:77-86; Coligan *et al.* (1991) *Current Protocols in Immunology* 13:12; Kwekkeboom *et al.* (1993) 30 *Immunology* 79:439-444; and U.S. Patent Nos. 5,674,492 and 5,847,082; herein incorporated by reference.

A representative assay to detect antagonistic anti-CD40 antibodies specific to the CD40-antigen epitopes identified herein is a "competitive binding assay."

Competitive binding assays are serological assays in which unknowns are detected and quantitated by their ability to inhibit the binding of a labeled known ligand to its specific antibody. This is also referred to as a competitive inhibition assay. In a representative competitive binding assay, labeled CD40 polypeptide is precipitated by 5 candidate antibodies in a sample, for example, in combination with monoclonal antibodies raised against one or more epitopes of the monoclonal antibodies of the invention. Anti-CD40 antibodies that specifically react with an epitope of interest can be identified by screening a series of antibodies prepared against a CD40 protein or fragment of the protein comprising the particular epitope of the CD40 protein of 10 interest. For example, for human CD40, epitopes of interest include epitopes comprising linear and/or nonlinear amino acid residues of the short isoform of human CD40 (see GenBank Accession No. NP_690593) set forth in Figure 4B (SEQ ID NO:10), encoded by the sequence set forth in Figure 4A (SEQ ID NO:9; see also GenBank Accession No. NM_152854), or of the long isoform of human CD40 (see 15 GenBank Accession Nos. CAA43045 and NP_001241) set forth in Figure 4D (SEQ ID NO:12), encoded by the sequence set forth in Figure 4C (SEQ ID NO:11; see GenBank Accession Nos. X60592 and NM_001250). Alternatively, competitive binding assays with previously identified suitable antagonist anti-CD40 antibodies could be used to select monoclonal antibodies comparable to the previously identified 20 antibodies.

Antibodies employed in such immunoassays may be labeled or unlabeled. Unlabeled antibodies may be employed in agglutination; labeled antibodies may be employed in a wide variety of assays, employing a wide variety of labels. Detection 25 of the formation of an antibody-antigen complex between an anti-CD40 antibody and an epitope of interest can be facilitated by attaching a detectable substance to the antibody. Suitable detection means include the use of labels such as radionuclides, enzymes, coenzymes, fluorescers, chemiluminescers, chromogens, enzyme substrates or co-factors, enzyme inhibitors, prosthetic group complexes, free radicals, particles, dyes, and the like. Examples of suitable enzymes include horseradish peroxidase, 30 alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein

isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material is luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S , or ^3H . Such labeled reagents may
5 be used in a variety of well-known assays, such as radioimmunoassays, enzyme immunoassays, e.g., ELISA, fluorescent immunoassays, and the like. See for example, U.S. Patent Nos. 3,766,162; 3,791,932; 3,817,837; and 4,233,402.

Any of the previously described antagonist anti-CD40 antibodies or antibody fragments thereof may be conjugated prior to use in the methods of the present
10 invention. Methods for producing conjugated antibodies are known in the art. Thus, the anti-CD40 antibody may be labeled using an indirect labeling or indirect labeling approach. By "indirect labeling" or "indirect labeling approach" is intended that a chelating agent is covalently attached to an antibody and at least one radionuclide is inserted into the chelating agent. See, for example, the chelating agents and
15 radionuclides described in Srivastava and Mease (1991) *Nucl. Med. Bio.* 18:589-603, herein incorporated by reference. Suitable labels include fluorophores, chromophores, radioactive atoms (particularly ^{32}P and ^{125}I), electron-dense reagents, enzymes, and ligands having specific binding partners. Enzymes are typically detected by their activity. For example, horseradish peroxidase is usually detected by
20 its ability to convert 3,3 ',5,5 '-tetramethylbenzidine (TMB) to a blue pigment, quantifiable with a spectrophotometer. "Specific binding partner" refers to a protein capable of binding a ligand molecule with high specificity, as for example in the case of an antigen and a monoclonal antibody specific thereto. Other specific binding partners include biotin and avidin or streptavidin, Ig G and protein A, and the
25 numerous receptor-ligand couples known in the art. It should be understood that the above description is not meant to categorize the various labels into distinct classes, as the same label may serve in several different modes. For example, ^{125}I may serve as a radioactive label or as an electron-dense reagent. HRP may serve as enzyme or as antigen for a mAb. Further, one may combine various labels for desired effect. For
30 example, mAbs and avidin also require labels in the practice of this invention: thus, one might label a mAb with biotin, and detect its presence with avidin labeled with ^{125}I , or with an anti-biotin mAb labeled with HRP. Other permutations and

possibilities will be readily apparent to those of ordinary skill in the art, and are considered as equivalents within the scope of the instant invention.

Alternatively, the anti-CD40 antibody may be labeled using "direct labeling" or a "direct labeling approach," where a radionuclide is covalently attached directly to an antibody (typically via an amino acid residue). Preferred radionuclides are provided in Srivagtava and Mease (1991) *supra*. The indirect labeling approach is particularly preferred. See also, for example, International Publication Nos. WO 00/52031 and WO 00/52473, where a linker is used to attach a radioactive label to antibodies; and the labeled forms of anti-CD40 antibodies described in U.S. Patent No. 6,015,542; herein incorporated by reference.

Further, an antibody (or fragment thereof) may be conjugated to a therapeutic moiety such as a cytotoxin, a therapeutic agent, or a radioactive metal ion or radioisotope. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., fludarabine, 2-chlorodeoxyadenosine, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa, chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine). Radioisotopes include, but are not limited to, I-131, I-123, I-125, Y-90, Re-188, Re-186, At-211, Cu-67, Bi-212, Bi-213, Pd-109, Tc-99, In-111, and the like. The conjugates of the invention can be used for modifying a given biological response; the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins

may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, interferon-alpha, interferon-beta, nerve growth factor, platelet derived growth factor, tissue plasminogen activator; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

Techniques for conjugating such therapeutic moiety to antibodies are well known. See, for example, Arnon *et al.* (1985) "Monoclonal Antibodies for Immunotargeting of Drugs in Cancer Therapy," in *Monoclonal Antibodies and Cancer Therapy*, ed. Reisfeld *et al.* (Alan R. Liss, Inc.), pp. 243-256; ed. Hellstrom *et al.* (1987) "Antibodies for Drug Delivery," in *Controlled Drug Delivery*, ed. Robinson *et al.* (2d ed; Marcel Dekker, Inc.), pp. 623-653; Thorpe (1985) "Antibody Carriers of Cytotoxic Agents in Cancer Therapy: A Review," in *Monoclonal Antibodies '84: Biological and Clinical Applications*, ed. Pinchera *et al.* pp. 475-506 (Editrice Kurtis, Milano, Italy, 1985); "Analysis, Results, and Future Prospective of the Therapeutic Use of Radiolabeled Antibody in Cancer Therapy," in *Monoclonal Antibodies for Cancer Detection and Therapy*, ed. Baldwin *et al.* (Academic Press, New York, 1985), pp. 303-316; and Thorpe *et al.* (1982) *Immunol. Rev.* 62:119-158.

Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described in U.S. Patent No. 4,676,980. In addition, linkers may be used between the labels and the antibodies of the invention (see U.S. Patent No. 4,831,175). Antibodies or, antigen-binding fragments thereof may be directly labeled with radioactive iodine, indium, yttrium, or other radioactive particle known in the art (U.S. Patent No. 5,595,721). Treatment may consist of a combination of treatment with conjugated and nonconjugated antibodies administered simultaneously or subsequently (WO 00/52031 and WO 00/52473).

Variants of Antagonist Anti-CD40 Antibodies

Suitable biologically active variants of the antagonist anti-CD40 antibodies can be used in the methods of the present invention. Such variants will retain the

desired binding properties of the parent antagonist anti-CD40 antibody. Methods for making antibody variants are generally available in the art.

For example, amino acid sequence variants of an antagonist anti-CD40 antibody, for example, the CHIR-5.9 or CHIR-12.12 monoclonal antibody described herein, can be prepared by mutations in the cloned DNA sequence encoding the antibody of interest. Methods for mutagenesis and nucleotide sequence alterations are well known in the art. See, for example, Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York); Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods Enzymol.* 10 154:367-382; Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, New York); U.S. Patent No. 4,873,192; and the references cited therein; herein incorporated by reference. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the polypeptide of interest may be found in the model of Dayhoff *et al.* (1978) in *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be preferred. Examples of conservative substitutions include, but are not limited to, Gly↔Ala, Val↔Ile↔Leu, Asp↔Glu, Lys↔Arg, Asn↔Gln, and Phe↔Trp↔Tyr.

In constructing variants of the antagonist anti-CD40 antibody polypeptide of interest, modifications are made such that variants continue to possess the desired activity, i.e., similar binding affinity and are capable of specifically binding to a human CD40 antigen expressed on the surface of a human cell, and being free of significant agonist activity but exhibiting antagonist activity when bound to a CD40 antigen on a human CD40-expressing cell. Obviously, any mutations made in the DNA encoding the variant polypeptide must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See EP Patent Application Publication No. 75,444.

In addition, the constant region of an antagonist anti-CD40 antibody can be mutated to alter effector function in a number of ways. For example, see U.S. Patent No. 6,737,056B1 and U.S. Patent Application Publication No. 2004/0132101A1, which disclose Fc mutations that optimize antibody binding to Fc receptors.

Preferably, variants of a reference antagonist anti-CD40 antibody have amino acid sequences that have at least 70% or 75% sequence identity, preferably at least 80% or 85% sequence identity, more preferably at least 90%, 91%, 92%, 93%, 94% or 95% sequence identity to the amino acid sequence for the reference antagonist anti-
5 CD40 antibody molecule, for example, the CHIR-5.9 or CHIR-12.12 monoclonal antibody described herein, or to a shorter portion of the reference antibody molecule. More preferably, the molecules share at least 96%, 97%, 98% or 99% sequence identity. For purposes of the present invention, percent sequence identity is determined using the Smith-Waterman homology search algorithm using an affine
10 gap search with a gap open penalty of 12 and a gap extension penalty of 2, BLOSUM matrix of 62. The Smith-Waterman homology search algorithm is taught in Smith and Waterman (1981) *Adv. Appl. Math.* 2:482-489. A variant may, for example, differ from the reference antagonist anti-CD40 antibody by as few as 1 to 15 amino acid residues, as few as 1 to 10 amino acid residues, such as 6-10, as few as 5, as few
15 as 4, 3, 2, or even 1 amino acid residue.

With respect to optimal alignment of two amino acid sequences, the contiguous segment of the variant amino acid sequence may have additional amino acid residues or deleted amino acid residues with respect to the reference amino acid sequence. The contiguous segment used for comparison to the reference amino acid
20 sequence will include at least 20 contiguous amino acid residues, and may be 30, 40, 50, or more amino acid residues. Corrections for sequence identity associated with conservative residue substitutions or gaps can be made (see Smith-Waterman homology search algorithm).

The precise chemical structure of a polypeptide capable of specifically binding
25 CD40 and retaining antagonist activity, particularly when bound to CD40 antigen on malignant B cells, depends on a number of factors. As ionizable amino and carboxyl groups are present in the molecule, a particular polypeptide may be obtained as an acidic or basic salt, or in neutral form. All such preparations that retain their biological activity when placed in suitable environmental conditions are included in
30 the definition of antagonist anti-CD40 antibodies as used herein. Further, the primary amino acid sequence of the polypeptide may be augmented by derivatization using sugar moieties (glycosylation) or by other supplementary molecules such as lipids,

phosphate, acetyl groups and the like. It may also be augmented by conjugation with saccharides. Certain aspects of such augmentation are accomplished through post-translational processing systems of the producing host; other such modifications may be introduced *in vitro*. In any event, such modifications are included in the definition 5 of an anti-CD40 antibody used herein so long as the antagonist properties of the anti-CD40 antibody are not destroyed. It is expected that such modifications may quantitatively or qualitatively affect the activity, either by enhancing or diminishing the activity of the polypeptide, in the various assays. Further, individual amino acid residues in the chain may be modified by oxidation, reduction, or other derivatization, 10 and the polypeptide may be cleaved to obtain fragments that retain activity. Such alterations that do not destroy antagonist activity do not remove the polypeptide sequence from the definition of anti-CD40 antibodies of interest as used herein.

The art provides substantial guidance regarding the preparation and use of 15 polypeptide variants. In preparing the anti-CD40 antibody variants, one of skill in the art can readily determine which modifications to the native protein nucleotide or amino acid sequence will result in a variant that is suitable for use as a therapeutically active component of a pharmaceutical composition used in the methods of the present invention.

20 **Methods of Therapy Using the Antagonist Anti-CD40 Antibodies of the Invention**

Methods of the invention are directed to the use of antagonist anti-CD40 antibodies to treat subjects (i.e., patients) having chronic lymphocytic leukemia (CLL), where the cells of this cancer express the CD40 antigen. By "CD40-expressing chronic lymphocytic leukemia cell" is intended CLL cells that express the 25 CD40 antigen. The successful treatment of CLL depends on how advanced the cancer is at the time of diagnosis, and whether the subject has or will undergo other methods of therapy in combination with anti-CD40 antibody administration. Methods for detecting CD40 expression in cells are well known in the art and include, but are not limited to, PCR techniques, immunohistochemistry, flow cytometry, Western blot, 30 ELISA, and the like.

A number of criteria can be used to classify stage of CLL. The methods of the present invention can be utilized to treat CLLs classified according to the Rai-Binet

classification system. In the Rai system, there are five stages: stage 0 wherein only lymphocytosis is present; stage I wherein lymphadenopathy is present; stage II wherein splenomegaly, lymphadenopathy, or both are present; stage III wherein anemia, organomegaly, or both are present (progression is defined by weight loss, 5 fatigue, fever, massive organomegaly, and a rapidly increasing lymphocyte count); and stage IV wherein anemia, thrombocytopenia, organomegaly, or a combination thereof are present. Under the Binet staging system there are only three categories: stage A wherein lymphocytosis is present and less than three lymph nodes are enlarged (this stage is inclusive of all Rai stage 0 patients, one-half of Rai stage I 10 patients, and one-third of Rai stage II patients); stage B wherein three or more lymph nodes are involved; and stage C wherein anemia or thrombocytopenia, or both are present. The Rai-Binet classification system can be combined with measurements of mutation of the immunoglobulin genes to provide a more accurate characterization of the state of the disease. The presence of mutated immunoglobulin genes correlates to 15 improved prognosis.

The methods of the present invention are applicable to treatment of CLL classified according to any of the foregoing criteria. Just as these criteria can be utilized to characterize progressive stages of the disease, these same criteria, i.e., anemia, lymphadenopathy, organomegaly, thrombocytopenia, and immunoglobulin 20 gene mutation, can be monitored to assess treatment efficacy.

"Treatment" is herein defined as the application or administration of an antagonist anti-CD40 antibody or antigen-binding fragment thereof to a subject, or application or administration of an antagonist anti-CD40 antibody or antigen-binding fragment thereof to an isolated tissue or cell line from a subject, where the subject has 25 CLL, a symptom associated with CLL, or a predisposition toward development of CLL, where the purpose is to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the CLL, any associated symptoms of CLL, or the predisposition toward the development of CLL. By "treatment" is also intended the application or administration of a pharmaceutical composition comprising an antagonist anti-CD40 30 antibodies or antigen-binding fragment thereof to a subject, or application or administration of a pharmaceutical composition comprising an antagonist anti-CD40 antibody or antigen-binding fragment thereof to an isolated tissue or cell line from a

subject, where the subject has CLL, a symptom associated with CLL, or a predisposition toward development of CLL, where the purpose is to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the CLL, any associated symptoms of CLL, or the predisposition toward the development of CLL.

5 By "anti-tumor activity" is intended a reduction in the rate of malignant CD40-expressing cell proliferation or accumulation, and hence a decline in growth rate of an existing tumor or in a tumor that arises during therapy, and/or destruction of existing neoplastic (tumor) cells or newly formed neoplastic cells, and hence a decrease in the overall size of a tumor during therapy. Therapy with at least one anti-CD40 antibody
10 (or antigen-binding fragment thereof) causes a physiological response that is beneficial with respect to treatment of CLL, where the disease comprises cells expressing the CD40 antigen. It is recognized that the methods of the invention may be useful in preventing further proliferation and outgrowths of CLL cells arising during therapy.

15 In accordance with the methods of the present invention, at least one antagonist anti-CD40 antibody (or antigen-binding fragment thereof) as defined elsewhere herein is used to promote a positive therapeutic response with respect to treatment or prevention of CLL. By "positive therapeutic response" with respect to cancer treatment is intended an improvement in the disease in association with the
20 anti-tumor activity of these antibodies or antigen-binding fragments thereof, and/or an improvement in the symptoms associated with the disease. That is, an anti-proliferative effect, the prevention of further tumor outgrowths, a reduction in tumor size, a reduction in the number of cancer (i.e., neoplastic) cells, an increase in neoplastic cell death, inhibition of neoplastic cell survival, an increased patient
25 survival rate, and/or a decrease in one or more symptoms mediated by stimulation of CD40-expressing cells can be observed. Thus, for example, an improvement in the disease may be characterized as a complete response. By "complete response" is intended an absence of clinically detectable disease with normalization of any previously abnormal radiographic studies, bone marrow, and cerebrospinal fluid
30 (CSF). Such a response must persist for at least one month following treatment according to the methods of the invention. Alternatively, an improvement in the disease may be categorized as being a partial response. By "partial response" is

intended at least about a 50% decrease in all measurable tumor burden (i.e., the number of tumor cells present in the subject) in the absence of new lesions and persisting for at least one month. Such a response is applicable to measurable tumors only.

5 Tumor response can be assessed for changes in tumor morphology (i.e., overall tumor burden, tumor size, and the like) using screening techniques such as magnetic resonance imaging (MRI) scan, x-radiographic imaging, computed tomographic (CT) scan, flow cytometry or fluorescence-activated cell sorter (FACS) analysis, bioluminescent imaging, for example, luciferase imaging, bone scan imaging, and tumor biopsy sampling including bone marrow aspiration (BMA). In addition to these positive therapeutic responses, the subject undergoing therapy with the antagonist anti-CD40 antibody or antigen-binding fragment thereof may experience the beneficial effect of an improvement in the symptoms associated with the disease.

10 By "therapeutically effective dose or amount" or "effective amount" is intended an amount of antagonist anti-CD40 antibody or antigen-binding fragment thereof that, when administered brings about a positive therapeutic response with respect to treatment of a subject with CLL. In some embodiments of the invention, a therapeutically effective dose of the anti-CD40 antibody or fragment thereof is in the range from about 0.01 mg/kg to about 40 mg/kg, from about 0.01 mg/kg to about 30 mg/kg, from about 0.1 mg/kg to about 30 mg/kg, from about 1 mg/kg to about 30 mg/kg, from about 3 mg/kg to about 30 mg/kg, from about 3 mg/kg to about 25 mg/kg, from about 3 mg/kg to about 20 mg/kg, from about 5 mg/kg to about 15 mg/kg, or from about 7 mg/kg to about 12 mg/kg. It is recognized that the method of treatment may comprise a single administration of a therapeutically effective dose or multiple administrations of a therapeutically effective dose of the antagonist anti-CD40 antibody or antigen-binding fragment thereof.

15 A further embodiment of the invention is the use of antagonist anti-CD40 antibodies for diagnostic monitoring of protein levels in tissue as part of a clinical testing procedure, e.g., to determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups,

fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S , or ^3H .

5 The antagonist anti-CD40 antibodies can be used in combination with known chemotherapeutics, alone or in combination with surgery or surgical procedures (e.g. splenectomy, hepatectomy, lymphadenectomy, leukapheresis, bone marrow transplantation, and the like), radiation therapy, chemotherapy, other anti-cancer monoclonal antibody therapy, steroids, IL-2 therapy, and interferon-alpha for the treatment of CLL. In this manner, the antagonist anti-CD40 antibodies described herein, or antigen-binding fragments thereof, are administered in combination with at least one other cancer therapy, including, but not limited to, surgery, radiation therapy, chemotherapy, other anti-cancer monoclonal antibody therapy (for example, alemtuzumab (Campath $^{\circledR}$), targeting the CD52 cell surface antigen on malignant B cells; rituximab (Rituxan $^{\circledR}$), targeting the CD20 cell surface antigen on malignant B cells, or other therapeutic anti-CD20 antibody, for example, the fully human antibody HuMax-CD20, R-1594, IMMU-106, TRU-015, AME-133, tositumomab/I-131 tositumomab (Bexxar $^{\circledR}$), ibritumomab tiuxetan (Zevalin $^{\circledR}$); or anti-CD23 antibody targeting the CD23 antigen on malignant B cells); interferon-alpha therapy, interleukin-2 (IL-2) therapy, therapy with IL-12, IL-15, or IL-21, or steroid therapy, where the additional cancer therapy is administered prior to, during, or subsequent to the anti-CD40 antibody therapy. Thus, where the combined therapies comprise administration of an anti-CD40 antibody or antigen-binding fragment thereof in combination with administration of another therapeutic agent, as with chemotherapy, radiation therapy, or therapy with interferon-alpha, IL-2, and/or steroids, the methods of the invention encompass coadministration, using separate formulations or a single pharmaceutical formulation, and consecutive administration in either order. Where

10 15 20 25 30

the methods of the present invention comprise combined therapeutic regimens, these therapies can be given simultaneously, i.e., the anti-CD40 antibody or antigen-binding fragment thereof is administered concurrently or within the same time frame as the other cancer therapy (i.e., the therapies are going on concurrently, but the anti-CD40 antibody or antigen-binding fragment thereof is not administered precisely at the same time as the other cancer therapy). Alternatively, the anti-CD40 antibody of the present invention or antigen-binding fragment thereof may also be administered prior to or subsequent to the other cancer therapy. Sequential administration of the different cancer therapies may be performed regardless of whether the treated subject responds to the first course of therapy to decrease the possibility of remission or relapse. Where the combined therapies comprise administration of the anti-CD40 antibody or antigen-binding fragment thereof in combination with administration of a cytotoxic agent, preferably the anti-CD40 antibody or antigen-binding fragment thereof is administered prior to administering the cytotoxic agent.

In some embodiments of the invention, the antagonist anti-CD40 antibodies described herein, or antigen-binding fragments thereof, are administered in combination with chemotherapy, and optionally in combination with autologous bone marrow transplantation, wherein the antibody and the chemotherapeutic agent(s) may be administered sequentially, in either order, or simultaneously (i.e., concurrently or within the same time frame). Examples of suitable chemotherapeutic agents include, but are not limited to, fludarabine, chlorambucil, vincristine, pentostatin, 2-chlorodeoxyadenosine (cladribine), cyclophosphamide, doxorubicin, and prednisone.

Thus, for example, in some embodiments, the antagonist anti-CD40 antibody, for example, the monoclonal antibody CHIR-12.12 or CHIR-5.9 or antigen-binding fragment thereof, is administered in combination with fludarabine. In one such embodiment, the antagonist anti-CD40 antibody is administered prior to administration of fludarabine. In alternative embodiments, the antagonist anti-CD40 antibody is administered after treatment with fludarabine. In yet other embodiments, the fludarabine is administered simultaneously with the antagonist anti-CD40 antibody.

In other embodiments of the invention, chlorambucil, an alkylating drug, is administered in combination with an antagonist anti-CD40 antibody described herein,

for example, the monoclonal antibody CHIR-12.12 or CHIR-5.9 or an antigen-binding fragment thereof. In one such embodiment, the antagonist anti-CD40 antibody is administered prior to administration of chlorambucil. In alternative embodiments, the antagonist anti-CD40 antibody is administered after treatment with 5 chlorambucil. In yet other embodiments, the chlorambucil is administered simultaneously with the antagonist anti-CD40 antibody.

In yet other embodiments, anthracycline-containing regimens such as CAP (cyclophosphamide, doxorubicin plus prednisone) and CHOP (cyclophosphamide, vincristine, prednisone plus doxorubicin) may be combined with administration of an 10 antagonist anti-CD40 antibody described herein, for example, the monoclonal antibody CHIR-12.12 or CHIR-5.9 or an antigen-binding fragment thereof. In one such embodiment, the antagonist anti-CD40 antibody is administered prior to administration of anthracycline-containing regimens. In other embodiments, the antagonist anti-CD40 antibody is administered after treatment with anthracycline- 15 containing regimens. In yet other embodiments, the anthracycline-containing regimen is administered simultaneously with the antagonist anti-CD40 antibody.

In alternative embodiments, an antagonist anti-CD40 antibody described herein, for example, the monoclonal antibody CHIR-12.12 or CHIR-5.9 or an antigen-binding fragment thereof, is administered in combination with alemtuzumab 20 (Campath[®]; distributed by Berlex Laboratories, Richmond, California).

Alemtuzumab is a recombinant humanized monoclonal antibody (Campath-1H) that targets the CD52 antigen expressed on malignant B cells. In one such embodiment, the antagonist anti-CD40 antibody is administered prior to administration of alemtuzumab. In other embodiments, the antagonist anti-CD40 antibody is 25 administered after treatment with alemtuzumab. In yet other embodiments, the alemtuzumab is administered simultaneously with the antagonist anti-CD40 antibody.

In other embodiments, the antagonist anti-CD40 antibodies described herein, for example, the monoclonal antibody CHIR-12.12 or CHIR-5.9 or antigen-binding fragment thereof, can be used in combination with another agent that has anti- 30 angiogenic properties, such as thalidomide, or interferon-alpha. These latter agents can be effective where a subject is resistant to first-line therapy. Alternatively, the

antagonist anti-CD40 antibodies can be administered to a subject in combination with high dose chemotherapy, alone or with autologous bone marrow transplantation.

In alternative embodiments, an antagonist anti-CD40 antibody described herein, for example, the monoclonal antibody CHIR-12.12 or CHIR-5.9 or an antigen-binding fragment thereof, can be used in combination with other immunotherapeutic agents, notably IL-2. IL-2, an agent known to expand the number of natural killer (NK) effector cells in treated patients, can be administered prior to, or concomitantly with, the antagonist anti-CD40 antibody of the invention. This expanded number of NK effector cells may lead to enhanced ADCC activity of the administered antagonist anti-CD40 antibody.

Further, combination therapy with two or more therapeutic agents and an antagonist anti-CD40 antibody described herein can also be used for treatment of CLL. Without being limiting, examples include triple combination therapy, where two chemotherapeutic agents are administered in combination with an antagonist anti-CD40 antibody described herein, and where a chemotherapeutic agent and another anti-cancer monoclonal antibody (for example, alemtuzumab; rituximab or other anti-CD20 antibody, for example, the fully human antibody HuMax-CD20, R-1594, IMMU-106, TRU-015, AME-133, tositumomab/I-131 tositumomab (Bexxar®), ibritumomab tiuxetan (Zevalin®); or anti-CD23 antibody) are administered in combination with an antagonist anti-CD40 antibody described herein. Examples of such combinations include, but are not limited to, combinations of fludarabine, cyclophosphamide, and the antagonist anti-CD40 antibody, for example, the monoclonal antibody CHIR-12.12 or CHIR-5.9 or an antigen-binding fragment thereof; and combinations of fludarabine, an anti-CD20 antibody, for example, rituximab (Rituxan®; IDEC Pharmaceuticals Corp., San Diego, California), and the antagonist anti-CD40 antibody, for example, the monoclonal antibody CHIR-12.12 or CHIR-5.9 or an antigen-binding fragment thereof.

Pharmaceutical Formulations and Modes of Administration

The antagonist anti-CD40 antibodies of this invention are administered at a concentration that is therapeutically effective to prevent or treat chronic lymphocytic leukemia. To accomplish this goal, the antibodies may be formulated using a variety

of acceptable excipients known in the art. Typically, the antibodies are administered by injection, for example, either intravenously, intraperitoneally, or subcutaneously. Methods to accomplish this administration are known to those of ordinary skill in the art. It may also be possible to obtain compositions that may be topically or orally administered, or which may be capable of transmission across mucous membranes.

Intravenous administration occurs preferably by infusion over a period of about 1 to about 10 hours, more preferably over about 1 to about 8 hours, even more preferably over about 2 to about 7 hours, still more preferably over about 4 to about 6 hours, depending upon the anti-CD40 antibody being administered. The initial infusion with the pharmaceutical composition may be given over a period of about 4 to about 6 hours with subsequent infusions delivered more quickly. Subsequent infusions may be administered over a period of about 1 to about 6 hours, including, for example, about 1 to about 4 hours, about 1 to about 3 hours, or about 1 to about 2 hours.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of possible routes of administration include parenteral, (e.g., intravenous (IV), intramuscular (IM), intradermal, subcutaneous (SC), or infusion), oral and pulmonary (e.g., inhalation), nasal, transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerin, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes, or multiple dose vials made of glass or plastic.

The antagonist anti-CD40 antibodies are typically provided by standard technique within a pharmaceutically acceptable buffer, for example, sterile saline, sterile buffered water, propylene glycol, combinations of the foregoing, etc. Methods

for preparing parenterally administrable agents are described in *Remington's Pharmaceutical Sciences* (18th ed.; Mack Publishing Company, Eaton, Pennsylvania, 1990), herein incorporated by reference. See also, for example, WO 98/56418, which describes stabilized antibody pharmaceutical formulations suitable for use in the 5 methods of the present invention.

The amount of at least one antagonist anti-CD40 antibody or fragment thereof to be administered is readily determined by one of ordinary skill in the art without undue experimentation. Factors influencing the mode of administration and the respective amount of at least one antagonist anti-CD40 antibody (or fragment thereof) 10 include, but are not limited to, the particular disease undergoing therapy, the severity of the disease, the history of the disease, and the age, height, weight, health, and physical condition of the individual undergoing therapy. Similarly, the amount of antagonist anti-CD40 antibody or fragment thereof to be administered will be dependent upon the mode of administration and whether the subject will undergo a 15 single dose or multiple doses of this anti-tumor agent. Generally, a higher dosage of anti-CD40 antibody or fragment thereof is preferred with increasing weight of the patient undergoing therapy. The dose of anti-CD40 antibody or fragment thereof to be administered is in the range from about 0.003 mg/kg to about 50 mg/kg, preferably in the range of 0.01 mg/kg to about 40 mg/kg. Thus, for example, the dose can be 20 0.01 mg/kg, 0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg, 0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg, 2.5 mg/kg, 3 mg/kg, 5 mg/kg, 7 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg, 25 mg/kg, 30 mg/kg, 35 mg/kg, 40 mg/kg, 45 mg/kg, or 50 mg/kg.

In another embodiment of the invention, the method comprises administration 25 of multiple doses of antagonist anti-CD40 antibody or fragment thereof. The method may comprise administration of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, or more therapeutically effective doses of a pharmaceutical composition comprising an antagonist anti-CD40 antibody or fragment thereof. The frequency and duration of administration of multiple doses of the pharmaceutical compositions comprising anti-CD40 antibody or fragment thereof can be readily determined by one of skill in the art 30 without undue experimentation. Moreover, treatment of a subject with a therapeutically effective amount of an antibody can include a single treatment or, preferably, can include a series of treatments. In a preferred example, a subject is

treated with antagonist anti-CD40 antibody or antigen-binding fragment thereof in the range of between about 0.1 to 20 mg/kg body weight, once per week for between about 1 to 10 weeks, preferably between about 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. Treatment 5 may occur annually to prevent relapse or upon indication of relapse. It will also be appreciated that the effective dosage of antibody or antigen-binding fragment thereof used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays as described herein. Thus, in one embodiment, the dosing regimen includes a 10 first administration of a therapeutically effective dose of at least one anti-CD40 antibody or fragment thereof on days 1, 7, 14, and 21 of a treatment period. In another embodiment, the dosing regimen includes a first administration of a therapeutically effective dose of at least one anti-CD40 antibody or fragment thereof on days 1, 2, 3, 4, 5, 6, and 7 of a week in a treatment period. Further embodiments 15 include a dosing regimen having a first administration of a therapeutically effective dose of at least one anti-CD40 antibody or fragment thereof on days 1, 3, 5, and 7 of a week in a treatment period; a dosing regimen including a first administration of a therapeutically effective dose of at least one anti-CD40 antibody or fragment thereof on days 1 and 3 of a week in a treatment period; and a preferred dosing regimen 20 including a first administration of a therapeutically effective dose of at least one anti-CD40 antibody or fragment thereof on day 1 of a week in a treatment period. The treatment period may comprise 1 week, 2 weeks, 3 weeks, a month, 3 months, 6 months, or a year. Treatment periods may be subsequent or separated from each other by a day, a week, 2 weeks, a month, 3 months, 6 months, or a year.

25 In some embodiments, the therapeutically effective doses of antagonist anti-CD40 antibody or antigen-binding fragment thereof ranges from about 0.003 mg/kg to about 50 mg/kg, from about 0.01 mg/kg to about 40 mg/kg, from about 0.01 mg/kg to about 30 mg/kg, from about 0.1 mg/kg to about 30 mg/kg, from about 0.5 mg/kg to about 30 mg/kg, from about 1 mg/kg to about 30 mg/kg, from about 3 mg/kg to about 30 mg/kg, from about 3 mg/kg to about 25 mg/kg, from about 3 mg/kg to about 20 mg/kg, from about 5 mg/kg to about 15 mg/kg, or from about 7 mg/kg to about 12 mg/kg. Thus, for example, the dose of any one antagonist anti-CD40 antibody or

antigen-binding fragment thereof, for example the anti-CD40 monoclonal antibody CHIR-12.12 or CHIR-5.9 or antigen-binding fragment thereof, can be 0.003 mg/kg, 0.01 mg/kg, 0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg, 0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg, 2.5 mg/kg, 3 mg/kg, 5 mg/kg, 7 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg, 25 mg/kg, 30 mg/kg, 35 mg/kg, 40 mg/kg, 45 mg/kg, 50 mg/kg, or other such doses falling within the range of about 0.003 mg/kg to about 50 mg/kg. The same therapeutically effective dose of an antagonist anti-CD40 antibody or antigen-binding fragment thereof can be administered throughout each week of antibody dosing.

5 Alternatively, different therapeutically effective doses of an antagonist anti-CD40 antibody or antigen-binding fragment thereof can be used over the course of a treatment period.

10 In other embodiments, the initial therapeutically effective dose of an antagonist anti-CD40 antibody or antigen-binding fragment thereof as defined elsewhere herein can be in the lower dosing range (i.e., about 0.003 mg/kg to about 20 mg/kg) with subsequent doses falling within the higher dosing range (i.e., from about 15 20 mg/kg to about 50 mg/kg).

15 In alternative embodiments, the initial therapeutically effective dose of an antagonist anti-CD40 antibody or antigen-binding fragment thereof as defined elsewhere herein can be in the upper dosing range (i.e., about 20 mg/kg to about 50 mg/kg) with subsequent doses falling within the lower dosing range (i.e., 0.003 mg/kg to about 20 mg/kg). Thus, in one embodiment, the initial therapeutically effective dose of the antagonist anti-CD40 antibody or antigen-binding fragment thereof is about 20 mg/kg to about 35 mg/kg, including about 20 mg/kg, about 25 mg/kg, about 20 30 mg/kg, and about 35 mg/kg, and subsequent therapeutically effective doses of the antagonist anti-CD40 antibody or antigen binding fragment thereof are about 5 mg/kg to about 15 mg/kg, including about 5 mg/kg, 8 mg/kg, 10 mg/kg, 12 mg/kg, and about 25 15 mg/kg.

20 In some embodiments of the invention, antagonist anti-CD40 antibody therapy is initiated by administering a “loading dose” of the antibody or antigen-binding fragment thereof to the subject in need of antagonist anti-CD40 antibody therapy. By “loading dose” is intended an initial dose of the antagonist anti-CD40 antibody or 30 antigen-binding fragment thereof that is administered to the subject, where the dose of

the antibody or antigen-binding fragment thereof administered falls within the higher dosing range (i.e., from about 20 mg/kg to about 50 mg/kg). The “loading dose” can be administered as a single administration, for example, a single infusion where the antibody or antigen-binding fragment thereof is administered IV, or as multiple 5 administrations, for example, multiple infusions where the antibody or antigen-binding fragment thereof is administered IV, so long as the complete “loading dose” is administered within about a 24-hour period. Following administration of the “loading dose,” the subject is then administered one or more additional therapeutically effective doses of the antagonist anti-CD40 antibody or antigen-binding fragment 10 thereof. Subsequent therapeutically effective doses can be administered, for example, according to a weekly dosing schedule, or once every two weeks, once every three weeks, or once every four weeks. In such embodiments, the subsequent therapeutically effective doses generally fall within the lower dosing range (i.e., 0.003 mg/kg to about 20 mg/kg).

15 Alternatively, in some embodiments, following the “loading dose,” the subsequent therapeutically effective doses of the antagonist anti-CD40 antibody or antigen-binding fragment thereof are administered according to a “maintenance schedule,” wherein the therapeutically effective dose of the antibody or antigen-binding fragment thereof is administered once a month, once every 6 weeks, once 20 every two months, once every 10 weeks, once every three months, once every 14 weeks, once every four months, once every 18 weeks, once every five months, once every 22 weeks, once every six months, once every 7 months, once every 8 months, once every 9 months, once every 10 months, once every 11 months, or once every 12 months. In such embodiments, the therapeutically effective doses of the antagonist 25 anti-CD40 antibody or antigen-binding fragment thereof fall within the lower dosing range (i.e., 0.003 mg/kg to about 20 mg/kg), particularly when the subsequent doses are administered at more frequent intervals, for example, once every two weeks to once every month, or within the higher dosing range (i.e., from about 20 mg/kg to about 50 mg/kg), particularly when the subsequent doses are administered at less 30 frequent intervals, for example, where subsequent doses are administered about one month to about 12 months apart.

The antagonist anti-CD40 antibodies present in the pharmaceutical compositions described herein for use in the methods of the invention may be native or obtained by recombinant techniques, and may be from any source, including mammalian sources such as, e.g., mouse, rat, rabbit, primate, pig, and human.

5 Preferably such polypeptides are derived from a human source, and more preferably are recombinant, human proteins from hybridoma cell lines.

The pharmaceutical compositions useful in the methods of the invention may comprise biologically active variants of the antagonist anti-CD40 antibodies of the invention. Such variants should retain the desired biological activity of the native 10 polypeptide such that the pharmaceutical composition comprising the variant polypeptide has the same therapeutic effect as the pharmaceutical composition comprising the native polypeptide when administered to a subject. That is, the variant anti-CD40 antibody will serve as a therapeutically active component in the pharmaceutical composition in a manner similar to that observed for the native 15 antagonist antibody, for example CHIR-5.9 or CHIR-12.12 as expressed by the hybridoma cell line 5.9 or 12.12, respectively. Methods are available in the art for determining whether a variant anti-CD40 antibody retains the desired biological activity, and hence serves as a therapeutically active component in the pharmaceutical composition. Biological activity of antibody variants can be measured using assays 20 specifically designed for measuring activity of the native antagonist antibody, including assays described in the present invention.

Any pharmaceutical composition comprising an antagonist anti-CD40 antibody having the binding properties described herein as the therapeutically active component can be used in the methods of the invention. Thus liquid, lyophilized, or 25 spray-dried compositions comprising one or more of the antagonist anti-CD40 antibodies of the invention may be prepared as an aqueous or nonaqueous solution or suspension for subsequent administration to a subject in accordance with the methods of the invention. Each of these compositions will comprise at least one of the antagonist anti-CD40 antibodies of the present invention as a therapeutically or 30 prophylactically active component. By "therapeutically or prophylactically active component" is intended the anti-CD40 antibody is specifically incorporated into the composition to bring about a desired therapeutic or prophylactic response with regard

to treatment, prevention, or diagnosis of a disease or condition within a subject when the pharmaceutical composition is administered to that subject. Preferably the pharmaceutical compositions comprise appropriate stabilizing agents, bulking agents, or both to minimize problems associated with loss of protein stability and biological activity during preparation and storage.

Formulants may be added to pharmaceutical compositions comprising an antagonist anti-CD40 antibody of the invention. These formulants may include, but are not limited to, oils, polymers, vitamins, carbohydrates, amine acids, salts, buffers, albumin, surfactants, or bulking agents. Preferably carbohydrates include sugar or sugar alcohols such as mono-, di-, or polysaccharides, or water soluble glucans. The saccharides or glucans can include fructose, glucose, mannose, sorbose, xylose, maltose, sucrose, dextran, pullulan, dextrin, α and β cyclodextrin, soluble starch, hydroxyethyl starch, and carboxymethylcellulose, or mixtures thereof. "Sugar alcohol" is defined as a C₄ to C₈ hydrocarbon having a hydroxyl group and includes galactitol, inositol, mannitol, xylitol, sorbitol, glycerol, and arabinol. These sugars or sugar alcohols may be used individually or in combination. The sugar or sugar alcohol concentration is between 1.0% and 7% w/v., more preferably between 2.0% and 6.0% w/v. Preferably amino acids include levorotary (L) forms of carnitine, arginine, and betaine; however, other amino acids may be added. Preferred polymers include polyvinylpyrrolidone (PVP) with an average molecular weight between 2,000 and 3,000, or polyethylene glycol (PEG) with an average molecular weight between 3,000 and 5,000. Surfactants that can be added to the formulation are shown in EP Nos. 270,799 and 268,110.

Additionally, antibodies can be chemically modified by covalent conjugation to a polymer to increase their circulating half-life, for example. Preferred polymers, and methods to attach them to peptides, are shown in U.S. Patent Nos. 4,766,106; 4,179,337; 4,495,285; and 4,609,546; which are all hereby incorporated by reference in their entireties. Preferred polymers are polyoxyethylated polyols and polyethylene glycol (PEG). PEG is soluble in water at room temperature and has the general formula: R(O--CH₂--CH₂)_nO--R where R can be hydrogen, or a protective group such as an alkyl or alkanol group. Preferably, the protective group has between 1 and 8 carbons, more preferably it is methyl. The symbol n is a positive integer, preferably

between 1 and 1,000, more preferably between 2 and 500. The PEG has a preferred average molecular weight between 1,000 and 40,000, more preferably between 2,000 and 20,000, most preferably between 3,000 and 12,000. Preferably, PEG has at least one hydroxy group, more preferably it is a terminal hydroxy group. It is this hydroxy
5 group which is preferably activated to react with a free amino group on the inhibitor. However, it will be understood that the type and amount of the reactive groups may be varied to achieve a covalently conjugated PEG/antibody of the present invention.

Water-soluble polyoxyethylated polyols are also useful in the present invention. They include polyoxyethylated sorbitol, polyoxyethylated glucose, polyoxyethylated
10 glycerol (POG), and the like. POG is preferred. One reason is because the glycerol backbone of polyoxyethylated glycerol is the same backbone occurring naturally in, for example, animals and humans in mono-, di-, triglycerides. Therefore, this branching would not necessarily be seen as a foreign agent in the body. The POG has a preferred molecular weight in the same range as PEG. The structure for POG is shown in Knauf
15 et al. (1988) *J. Bio. Chem.* 263:15064-15070, and a discussion of POG/IL-2 conjugates is found in U.S. Patent No. 4,766,106, both of which are hereby incorporated by reference in their entireties.

Another drug delivery system for increasing circulatory half-life is the liposome. Methods of preparing liposome delivery systems are discussed in Gabizon
20 et al. (1982) *Cancer Research* 42:4734; Cafiso (1981) *Biochem Biophys Acta* 649:129; and Szoka (1980) *Ann. Rev. Biophys. Eng.* 9:467. Other drug delivery systems are known in the art and are described in, e.g., Poznansky et al. (1980) *Drug Delivery Systems* (R.L. Juliano, ed., Oxford, N.Y.) pp. 253-315; Poznansky (1984) *Pharm Revs* 36:277.

25 The formulators to be incorporated into a pharmaceutical composition should provide for the stability of the antagonist anti-CD40 antibody or antigen-binding fragment thereof. That is, the antagonist anti-CD40 antibody or antigen-binding fragment thereof should retain its physical and/or chemical stability and have the desired biological activity, i.e., one or more of the antagonist activities defined herein
30 above, including, but not limited to, inhibition of immunoglobulin secretion by normal human peripheral B cells stimulated by T cells; inhibition of survival and/or proliferation of normal human peripheral B cells stimulated by Jurkat T cells;

inhibition of survival and/or proliferation of normal human peripheral B cells stimulated by CD40L-expressing cells or soluble CD40 ligand (sCD40L); inhibition of "survival" anti-apoptotic intracellular signals in any cell stimulated by sCD40L or solid-phase CD40L; inhibition of CD40 signal transduction in any cell upon ligation 5 with sCD40L or solid-phase CD40L; and inhibition of proliferation of human malignant B cells as noted elsewhere herein.

Methods for monitoring protein stability are well known in the art. See, for example, Jones (1993) *Adv. Drug Delivery Rev.* 10:29-90; Lee, ed. (1991) *Peptide and Protein Drug Delivery* (Marcel Dekker, Inc., New York, New York); and the stability 10 assays disclosed herein below. Generally, protein stability is measured at a chosen temperature for a specified period of time. In preferred embodiments, a stable antibody pharmaceutical formulation provides for stability of the antagonist anti-CD40 antibody or antigen-binding fragment thereof when stored at room temperature (about 25°C) for at least 1 month, at least 3 months, or at least 6 months, and/or is 15 stable at about 2-8°C for at least 6 months, at least 9 months, at least 12 months, at least 18 months, at least 24 months.

A protein such as an antibody, when formulated in a pharmaceutical composition, is considered to retain its physical stability at a given point in time if it shows no visual signs (i.e., discoloration or loss of clarity) or measurable signs (for 20 example, using size-exclusion chromatography (SEC) or UV light scattering) of precipitation, aggregation, and/or denaturation in that pharmaceutical composition. With respect to chemical stability, a protein such as an antibody, when formulated in a pharmaceutical composition, is considered to retain its chemical stability at a given point in time if measurements of chemical stability are indicative that the protein (i.e., 25 antibody) retains the biological activity of interest in that pharmaceutical composition. Methods for monitoring changes in chemical stability are well known in the art and include, but are not limited to, methods to detect chemically altered forms of the protein such as result from clipping, using, for example, SDS-PAGE, SEC, and/or matrix-assisted laser desorption ionization/time of flight mass spectrometry; and 30 degradation associated with changes in molecular charge (for example, associated with deamidation), using, for example, ion-exchange chromatography. See, for example, the methods disclosed herein below.

An antagonist anti-CD40 antibody or antigen-binding fragment thereof, when formulated in a pharmaceutical composition, is considered to retain a desired biological activity at a given point in time if the desired biological activity at that time is within about 30%, preferably within about 20% of the desired biological activity

5 exhibited at the time the pharmaceutical composition was prepared as determined in a suitable assay for the desired biological activity. Assays for measuring the desired biological activity of the antagonist anti-CD40 antibodies disclosed herein, and antigen-binding fragments thereof, can be performed as described in the Examples herein. See also the assays described in Schultze *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 92:8200-8204; Denton *et al.* (1998) *Pediatr. Transplant.* 2:6-15; Evans *et al.* (2000) *J. Immunol.* 164:688-697; Noelle (1998) *Agents Actions Suppl.* 49:17-22; Lederman *et al.* (1996) *Curr. Opin. Hematol.* 3:77-86; Coligan *et al.* (1991) *Current Protocols in Immunology* 13:12; Kwekkeboom *et al.* (1993) *Immunology* 79:439-444; and U.S. Patent Nos. 5,674,492 and 5,847,082; herein incorporated by reference.

10 15 In some embodiments of the invention, the antagonist anti-CD40 antibody, for example, the CHIR-12.12 or CHIR-5.9 monoclonal antibody, or antigen-binding fragment thereof is formulated in a liquid pharmaceutical formulation. The antagonist anti-CD40 antibody or antigen binding fragment thereof can be prepared using any method known in the art, including those methods disclosed herein above. In one embodiment, the antagonist anti-CD40 antibody, for example, the CHIR-12.12 or CHIR-5.9 monoclonal antibody, or antigen-binding fragment thereof is recombinantly produced in a CHO cell line.

20 25 Following its preparation and purification, the antagonist anti-CD40 antibody or antigen-binding fragment thereof can be formulated as a liquid pharmaceutical formulation in the manner set forth herein. Where the antagonist anti-CD40 antibody or antigen-binding fragment thereof is to be stored prior to its formulation, it can be frozen, for example, at $\leq -20^{\circ}\text{C}$, and then thawed at room temperature for further formulation. The liquid pharmaceutical formulation comprises a therapeutically effective amount of the antagonist anti-CD40 antibody or antigen-binding fragment thereof. The amount of antibody or antigen-binding fragment thereof present in the formulation takes into consideration the route of administration and desired dose volume.

30

In this manner, the liquid pharmaceutical composition comprises the antagonist anti-CD40 antibody, for example, the CHIR-12.12 or CHIR-5.9 antibody, or antigen-binding fragment thereof at a concentration of about 0.1 mg/ml to about 50.0 mg/ml, about 0.5 mg/ml to about 40.0 mg/ml, about 1.0 mg/ml to about 30.0 mg/ml, about 5.0 mg/ml to about 25.0 mg/ml, about 5.0 mg/ml to about 20.0 mg/ml, or about 15.0 mg/ml to about 25.0 mg/ml. In some embodiments, the liquid pharmaceutical composition comprises the antagonist anti-CD40 antibody or antigen-binding fragment thereof at a concentration of about 0.1 mg/ml to about 5.0 mg/ml, about 5.0 mg/ml to about 10.0 mg/ml, about 10.0 mg/ml to about 15.0 mg/ml, about 15.0 mg/ml to about 20.0 mg/ml, about 20.0 mg/ml to about 25.0 mg/ml, about 25.0 mg/ml to about 30.0 mg/ml, about 30.0 mg/ml to about 35.0 mg/ml, about 35.0 mg/ml to about 40.0 mg/ml, about 40.0 mg/ml to about 45.0 mg/ml, or about 45.0 mg/ml to about 50.0 mg/ml. In other embodiments, the liquid pharmaceutical composition comprises the antagonist anti-CD40 antibody or antigen-binding fragment thereof at a concentration of about 15.0 mg/ml, about 16.0 mg/ml, about 17.0 mg/ml, about 18.0 mg/ml, about 19.0 mg/ml, about 20.0 mg/ml, about 21.0 mg/ml, about 22.0 mg/ml, about 23.0 mg/ml, about 24.0 mg/ml, or about 25.0 mg/ml. The liquid pharmaceutical composition comprises the antagonist anti-CD40 antibody, for example, the CHIR-12.12 or CHIR-5.9 antibody, or antigen-binding fragment thereof and a buffer that maintains the pH of the formulation in the range of about pH 5.0 to about pH 7.0, including about pH 5.0, 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9, 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, 7.0, and other such values within the range of about pH 5.0 to about pH 7.0. In some embodiments, the buffer maintains the pH of the formulation in the range of about pH 5.0 to about pH 6.5, about pH 5.0 to about pH 6.0, about pH 5.0 to about pH 5.5, about pH 5.5 to about 7.0, about pH 5.5 to about pH 6.5, or about pH 5.5 to about pH 6.0.

Any suitable buffer that maintains the pH of the liquid anti-CD40 antibody formulation in the range of about pH 5.0 to about pH 7.0 can be used in the formulation, so long as the physicochemical stability and desired biological activity of the antibody are retained as noted herein above. Suitable buffers include, but are not limited to, conventional acids and salts thereof, where the counter ion can be, for example, sodium, potassium, ammonium, calcium, or magnesium. Examples of

conventional acids and salts thereof that can be used to buffer the pharmaceutical liquid formulation include, but are not limited to, succinic acid or succinate, citric acid or citrate, acetic acid or acetate, tartaric acid or tartarate, phosphoric acid or phosphate, gluconic acid or gluconate, glutamic acid or glutamate, aspartic acid or aspartate, maleic acid or maleate, and malic acid or malate buffers. The buffer concentration within the formulation can be from about 1 mM to about 50 mM, including about 1 mM, 2 mM, 5 mM, 8 mM, 10 mM, 15 mM, 20 mM, 25 mM, 30 mM, 35 mM, 40 mM, 45 mM, 50 mM, or other such values within the range of about 1 mM to about 50 mM. In some embodiments, the buffer concentration within the formulation is from about 5 mM to about 15 mM, including about 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, 10 mM, 11 mM, 12 mM, 13 mM, 14 mM, 15 mM, or other such values within the range of about 5 mM to about 15 mM.

In some embodiments of the invention, the liquid pharmaceutical formulation comprises a therapeutically effective amount of the antagonist anti-CD40 antibody, for example, the CHIR-12.12 or CHIR-5.9 monoclonal antibody, or antigen-binding fragment thereof and succinate buffer or citrate buffer at a concentration that maintains the pH of the formulation in the range of about pH 5.0 to about pH 7.0, preferably about pH 5.0 to about pH 6.5. By “succinate buffer” or “citrate buffer” is intended a buffer comprising a salt of succinic acid or a salt of citric acid, respectively. In a preferred embodiment, the succinate or citrate counterion is the sodium cation, and thus the buffer is sodium succinate or sodium citrate, respectively. However, any cation is expected to be effective. Other possible succinate or citrate cations include, but are not limited to, potassium, ammonium, calcium, and magnesium. As noted above, the succinate or citrate buffer concentration within the formulation can be from about 1 mM to about 50 mM, including about 1 mM, 2 mM, 5 mM, 8 mM, 10 mM, 15 mM, 20 mM, 25 mM, 30 mM, 35 mM, 40 mM, 45 mM, 50 mM, or other such values within the range of about 1 mM to about 50 mM. In some embodiments, the buffer concentration within the formulation is from about 5 mM to about 15 mM, including about 5 mM, 6 mM, 7 mM, 8 mM, 9 mM, 10 mM, 11 mM, 12 mM, 13 mM, 14 mM, or about 15 mM. In other embodiments, the liquid pharmaceutical formulation comprises the antagonist anti-CD40 antibody, for example, the CHIR-12.12 or CHIR-5.9 monoclonal antibody, or antigen-binding

fragment thereof at a concentration of about 0.1 mg/ml to about 50.0 mg/ml, or about 5.0 mg/ml to about 25.0 mg/ml, and succinate or citrate buffer, for example, sodium succinate or sodium citrate buffer, at a concentration of about 1 mM to about 20 mM, about 5 mM to about 15 mM, preferably about 10 mM.

5 Where it is desirable for the liquid pharmaceutical formulation to be near isotonic, the liquid pharmaceutical formulation comprising a therapeutically effective amount of the antagonist anti-CD40 antibody, for example, the CHIR-12.12 or CHIR-5.9 monoclonal antibody, or antigen-binding fragment thereof, and a buffer to maintain the pH of the formulation within the range of about pH 5.0 to about pH 7.0
10 can further comprise an amount of an isotonizing agent sufficient to render the formulation near isotonic. By "near isotonic" is intended the aqueous formulation has an osmolarity of about 240 mmol/kg to about 360 mmol/kg, preferably about 240 to about 340 mmol/kg, more preferably about 250 to about 330 mmol/kg, even more preferably about 260 to about 320 mmol/kg, still more preferably about 270 to about
15 310 mmol/kg. Methods of determining the isotonicity of a solution are known to those skilled in the art. See, for example, Setnikar *et al.* (1959) *J. Am. Pharm. Assoc.* 48:628.

Those skilled in the art are familiar with a variety of pharmaceutically acceptable solutes useful in providing isotonicity in pharmaceutical compositions.
20 The isotonizing agent can be any reagent capable of adjusting the osmotic pressure of the liquid pharmaceutical formulation of the present invention to a value nearly equal to that of a body fluid. It is desirable to use a physiologically acceptable isotonizing agent. Thus, the liquid pharmaceutical formulation comprising a therapeutically effective amount of the antagonist anti-CD40 antibody, for example, the CHIR-12.12 or CHIR-5.9 monoclonal antibody, or antigen-binding fragment thereof, and a buffer to maintain the pH of the formulation within the range of about pH 5.0 to about pH
25 7.0, can further comprise components that can be used to provide isotonicity, for example, sodium chloride; amino acids such as alanine, valine, and glycine; sugars and sugar alcohols (polyols), including, but not limited to, glucose, dextrose, fructose, sucrose, maltose, mannitol, trehalose, glycerol, sorbitol, and xylitol; acetic acid, other organic acids or their salts, and relatively minor amounts of citrates or phosphates.
30

The ordinary skilled person would know of additional agents that are suitable for providing optimal tonicity of the liquid formulation.

In some preferred embodiments, the liquid pharmaceutical formulation comprising a therapeutically effective amount of the antagonist anti-CD40 antibody, for example, the CHIR-12.12 or CHIR-5.9 monoclonal antibody, or antigen-binding fragment thereof, and a buffer to maintain the pH of the formulation within the range of about pH 5.0 to about pH 7.0, further comprises sodium chloride as the isotonizing agent. The concentration of sodium chloride in the formulation will depend upon the contribution of other components to tonicity. In some embodiments, the concentration of sodium chloride is about 50 mM to about 300 mM, about 50 mM to about 250 mM, about 50 mM to about 200 mM, about 50 mM to about 175 mM, about 50 mM to about 150 mM, about 75 mM to about 175 mM, about 75 mM to about 150 mM, about 100 mM to about 175 mM, about 100 mM to about 200 mM, about 100 mM to about 150 mM, about 125 mM to about 175 mM, about 125 mM to about 150 mM, about 130 mM to about 170 mM, about 130 mM to about 160 mM, about 135 mM to about 155 mM, about 140 mM to about 155 mM, or about 145 mM to about 155 mM. In one such embodiment, the concentration of sodium chloride is about 150 mM. In other such embodiments, the concentration of sodium chloride is about 150 mM, the buffer is sodium succinate or sodium citrate buffer at a concentration of about 5 mM to about 15 mM, the liquid pharmaceutical formulation comprises a therapeutically effective amount of the antagonist anti-CD40 antibody, for example, the CHIR-12.12 or CHIR-5.9 monoclonal antibody, or antigen-binding fragment thereof, and the formulation has a pH of about pH 5.0 to about pH 7.0, about pH 5.0 to about pH 6.0, or about pH 5.5 to about pH 6.5. In other embodiments, the liquid pharmaceutical formulation comprises the antagonist anti-CD40 antibody, for example, the CHIR-12.12 or CHIR-5.9 monoclonal antibody, or antigen-binding fragment thereof, at a concentration of about 0.1 mg/ml to about 50.0 mg/ml or about 5.0 mg/ml to about 25.0 mg/ml, about 150 mM sodium chloride, and about 10 mM sodium succinate or sodium citrate, at a pH of about pH 5.5.

Protein degradation due to freeze thawing or mechanical shearing during processing of a liquid pharmaceutical formulations of the present invention can be inhibited by incorporation of surfactants into the formulation in order to lower the

surface tension at the solution-air interface. Thus, in some embodiments, the liquid pharmaceutical formulation comprises a therapeutically effective amount of the antagonist anti-CD40 antibody, for example, the CHIR-12.12 or CHIR-5.9 monoclonal antibody, or antigen-binding fragment thereof, a buffer to maintain the pH of the formulation within the range of about pH 5.0 to about pH 7.0, and further comprises a surfactant. In other embodiments, the liquid pharmaceutical formulation comprises a therapeutically effective amount of the antagonist anti-CD40 antibody, for example, the CHIR-12.12 or CHIR-5.9 monoclonal antibody, or antigen-binding fragment thereof, a buffer to maintain the pH of the formulation within the range of about pH 5.0 to about pH 7.0, an isotonizing agent such as sodium chloride at a concentration of about 50 mM to about 300 mM, and further comprises a surfactant.

Typical surfactants employed are nonionic surfactants, including polyoxyethylene sorbitol esters such as polysorbate 80 (Tween 80) and polysorbate 20 (Tween 20); polyoxypropylene-polyoxyethylene esters such as Pluronic F68; polyoxyethylene alcohols such as Brij 35; simethicone; polyethylene glycol such as PEG400; lysophosphatidylcholine; and polyoxyethylene-p-t-octylphenol such as Triton X-100. Classic stabilization of pharmaceuticals by surfactants or emulsifiers is described, for example, in Levine *et al.* (1991) *J. Parenteral Sci. Technol.* 45(3):160-165, herein incorporated by reference. A preferred surfactant employed in the practice of the present invention is polysorbate 80. Where a surfactant is included, it is typically added in an amount from about 0.001 % to about 1.0% (w/v), about 0.001% to about 0.5%, about 0.001% to about 0.4%, about 0.001% to about 0.3%, about 0.001% to about 0.2%, about 0.005% to about 0.5%, about 0.005% to about 0.2%, about 0.01% to about 0.5%, about 0.01% to about 0.2%, about 0.03% to about 0.5%, about 0.03% to about 0.3%, about 0.05% to about 0.5%, or about 0.05% to about 0.2%.

Thus, in some embodiments, the liquid pharmaceutical formulation comprises a therapeutically effective amount of the antagonist anti-CD40 antibody, for example, the CHIR-12.12 or CHIR-5.9 monoclonal antibody, or antigen-binding fragment thereof, the buffer is sodium succinate or sodium citrate buffer at a concentration of about 1 mM to about 50 mM, about 5 mM to about 25 mM, or about 5 mM to about 15 mM; the formulation has a pH of about pH 5.0 to about pH 7.0, about pH 5.0 to

about pH 6.0, or about pH 5.5 to about pH 6.5; and the formulation further comprises a surfactant, for example, polysorbate 80, in an amount from about 0.001% to about 1.0% or about 0.001% to about 0.5%. Such formulations can optionally comprise an isotonizing agent, such as sodium chloride at a concentration of about 50 mM to about 5 300 mM, about 50 mM to about 200 mM, or about 50 mM to about 150 mM. In other embodiments, the liquid pharmaceutical formulation comprises the antagonist anti-CD40 antibody, for example, the CHIR-12.12 or CHIR-5.9 monoclonal antibody, or antigen-binding fragment thereof, at a concentration of about 0.1 mg/ml to about 50.0 mg/ml or about 5.0 mg/ml to about 25.0 mg/ml, including about 20.0 mg/ml; 10 about 50 mM to about 200 mM sodium chloride, including about 150 mM sodium chloride; sodium succinate or sodium citrate at about 5 mM to about 20 mM, including about 10 mM sodium succinate or sodium citrate; sodium chloride at a concentration of about 50 mM to about 200 mM, including about 150 mM; and optionally a surfactant, for example, polysorbate 80, in an amount from about 0.001% 15 to about 1.0%, including about 0.001% to about 0.5%; where the liquid pharmaceutical formulation has a pH of about pH 5.0 to about pH 7.0, about pH 5.0 to about pH 6.0, about pH 5.0 to about pH 5.5, about pH 5.5 to about pH 6.5, or about pH 5.5 to about pH 6.0.

The liquid pharmaceutical formulation can be essentially free of any 20 preservatives and other carriers, excipients, or stabilizers noted herein above. Alternatively, the formulation can include one or more preservatives, for example, antibacterial agents, pharmaceutically acceptable carriers, excipients, or stabilizers described herein above provided they do not adversely affect the physicochemical stability of the antagonist anti-CD40 antibody or antigen-binding fragment thereof. 25 Examples of acceptable carriers, excipients, and stabilizers include, but are not limited to, additional buffering agents, co-solvents, surfactants, antioxidants including ascorbic acid and methionine, chelating agents such as EDTA, metal complexes (for example, Zn-protein complexes), and biodegradable polymers such as polyesters. A thorough discussion of formulation and selection of pharmaceutically acceptable 30 carriers, stabilizers, and isomolutes can be found in *Remington's Pharmaceutical Sciences* (18th ed.; Mack Publishing Company, Eaton, Pennsylvania, 1990), herein incorporated by reference.

After the liquid pharmaceutical formulation or other pharmaceutical composition described herein is prepared, it can be lyophilized to prevent degradation. Methods for lyophilizing liquid compositions are known to those of ordinary skill in the art. Just prior to use, the composition may be reconstituted with a sterile diluent

5 (Ringer's solution, distilled water, or sterile saline, for example) that may include additional ingredients. Upon reconstitution, the composition is preferably administered to subjects using those methods that are known to those skilled in the art.

10 Use of Antagonist Anti-CD40 Antibodies in the Manufacture of Medicaments

The present invention also provides for the use of an antagonist anti-CD40 antibody or antigen-binding fragment thereof in the manufacture of a medicament for treating CLL in a subject, wherein the medicament is coordinated with treatment with at least one other cancer therapy. By "coordinated" is intended

15 the medicament is to be used either prior to, during, or after treatment of the subject with at least one other cancer therapy. Examples of other cancer therapies include, but are not limited to, those described herein above, i.e., surgery; radiation therapy; chemotherapy, optionally in combination with autologous bone marrow transplant, where Examples of other cancer therapies include, but are not limited to, surgery;

20 radiation therapy; chemotherapy, optionally in combination with autologous bone marrow transplant, where suitable chemotherapeutic agents include, but are not limited to, fludarabine or fludarabine phosphate, chlorambucil, vincristine, pentostatin, 2-chlorodeoxyadenosine (cladribine), cyclophosphamide, doxorubicin, prednisone, and combinations thereof, for example, anthracycline-containing

25 regimens such as CAP (cyclophosphamide, doxorubicin plus prednisone), CHOP (cyclophosphamide, vincristine, prednisone plus doxorubicin), VAD (vincristine, doxorubicin, plus dexamethasone), MP (melphalan plus prednisone), and other cytotoxic and/or therapeutic agents used in chemotherapy such as mitoxantrone, daunorubicin, idarubicin, asparaginase, and antimetabolites, including, but not limited

30 to, cytarabine, methotrexate, 5-fluorouracil decarbazine, 6-thioguanine, 6-mercaptopurine, and nelarabine; other anti-cancer monoclonal antibody therapy (for example, alemtuzumab (Campath[®]) or other anti-CD52 antibody targeting the CD52 cell-surface glycoprotein on malignant B cells; rituximab (Rituxan[®]), the fully human

antibody HuMax-CD20, R-1594, IMMU-106, TRU-015, AME-133, tositumomab/I-131 tositumomab (Bexxar®), ibritumomab tiuxetan (Zevalin®), or any other therapeutic anti-CD20 antibody targeting the CD20 antigen on malignant B cells; anti-CD19 antibody (for example, MT103, a bispecific antibody); anti-CD22 antibody
5 (for example, the humanized monoclonal antibody epratuzumab); bevacizumab (Avastin®) or other anti-cancer antibody targeting human vascular endothelial growth factor; anti-CD22 antibody targeting the CD22 antigen on malignant B cells (for example, the monoclonal antibody BL-22, an alphaCD22 toxin); α-M-CSF antibody targeting macrophage colony stimulating factor; antibodies targeting the receptor
10 activator of nuclear factor-kappaB (RANK) and its ligand (RANKL); anti-CD23 antibody targeting the CD23 antigen on malignant B cells (for example, IDEC-152); anti-CD38 antibody targeting the CD38 antigen on malignant B cells; antibodies targeting major histocompatibility complex class II receptors (anti-MHC antibodies) expressed on malignant B cells; other anti-CD40 antibodies (for example, SGN-40)
15 targeting the CD40 antigen on malignant B cells; and antibodies targeting tumor necrosis factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) (for example, the agonistic human monoclonal antibody HGS-ETR1) expressed on a number of tumors of hematopoietic origin); small molecule-based cancer therapy, including, but not limited to, microtubule and/or topoisomerase inhibitors (for
20 example, the mitotic inhibitor dolastatin and dolastatin analogues; the tubulin-binding agent T900607; XL119; and the topoisomerase inhibitor aminocamptothecin), SDX-105 (bendamustine hydrochloride), ixabepilone (an epothilone analog, also referred to as BMS-247550), protein kinase C inhibitors, for example, midostaurin ((PKC-412, CGP 41251, N-benzoylstaurosporine), pixantrone, eloxatin (an antineoplastic agent),
25 ganite (gallium nitrate), Thalomid® (thalidomide), immunomodulatory derivatives of thalidomide (for example, revlimid (formerly revimid)), Affinitak™ (antisense inhibitor of protein kinase C-alpha), SDX-101 (R-etodolac, inducing apoptosis of malignant lymphocytes), second-generation purine nucleoside analogs such as clofarabine, inhibitors of production of the protein Bcl-2 by cancer cells (for example,
30 the antisense agents oblimersen and Genasense®), proteasome inhibitors (for example, Velcade™ (bortezomib)), small molecule kinase inhibitors (for example, CHIR-258), small molecule VEGF inhibitors (for example, ZD-6474), small molecule inhibitors

of heat shock protein (HSP) 90 (for example, 17-AAG), small molecule inhibitors of histone deacetylases (for example, hybrid/polar cytodifferentiation HPC) agents such as suberanilohydroxamic acid (SAHA), and FR-901228) and apoptotic agents such as Trisenox® (arsenic trioxide) and Xcytrin® (motexafin gadolinium); vaccine
5 /immunotherapy-based cancer therapies, including, but not limited to, vaccine approaches (for example, Id-KLH, oncophage, vita lethine), personalized immunotherapy or active idiotype immunotherapy (for example, MyVax® Personalized Immunotherapy, formally designated GTOP-99), Promune® (CpG 7909, a synthetic agonist for toll-like receptor 9 (TLR9)), interferon-alpha therapy,
10 interleukin-2 (IL-2) therapy, IL-12 therapy; IL-15 therapy, and IL-21 therapy; steroid therapy; or other cancer therapy; where treatment with the additional cancer therapy, or additional cancer therapies, occurs prior to, during, or subsequent to treatment of the subject with the medicament comprising the antagonist anti-CD40 antibody or antigen-binding fragment thereof, as noted herein above. In one such embodiment,
15 the present invention provides for the use of the monoclonal antibody CHIR-12.12 or CHIR-5.9 in the manufacture of a medicament for treating CLL in a subject, wherein the medicament is coordinated with treatment with at least one other cancer therapy as noted herein above.

Thus, for example, in some embodiments, the invention provides for the use of
20 the monoclonal antibody CHIR-12.12 or CHIR-5.9, or antigen-binding fragment thereof, in the manufacture of a medicament for treating CLL in a subject, wherein the medicament is coordinated with treatment with chemotherapy, where the chemotherapy is selected from the group consisting of fludarabine, chlorambucil, vincristine, pentostatin, 2-chlorodeoxyadenosine (cladribine), cyclophosphamide,
25 doxorubicin, and prednisone, and anthracycline-containing regimens such as CAP (cyclophosphamide, doxorubicin plus prednisone) and CHOP (cyclophosphamide, vincristine, prednisone plus doxorubicin), and any combinations thereof; wherein the medicament is to be used either prior to, during, or after treatment of the subject with the other cancer therapy or, in the case of multiple combination therapies, either prior
30 to, during, or after treatment of the subject with the other cancer therapies.

In other embodiments, the invention provides for the use of the monoclonal antibody CHIR-12.12 or CHIR-5.9, or antigen-binding fragment thereof, in the

manufacture of a medicament for treating multiple myeloma in a subject, wherein the medicament is coordinated with treatment with at least one other anti-cancer antibody selected from the group consisting of alemtuzumab (Campath®) or other anti-CD52 antibody targeting the CD52 cell-surface glycoprotein on malignant B cells; rituximab 5 (Rituxan®), the fully human antibody HuMax-CD20, R-1594, IMMU-106, TRU-015, AME-133, tositumomab/I-131 tositumomab (Bexxar®), ibritumomab tiuxetan (Zevalin®), or any other therapeutic anti-CD20 antibody targeting the CD20 antigen on malignant B cells; anti-CD23 antibody targeting the CD23 antigen on malignant B cells (for example, IDEC-152); and anti-CD22 antibody targeting the CD22 antigen 10 on malignant B cells (for example, the monoclonal antibody BL-22, an alphaCD22 toxin), and any combinations thereof; wherein the medicament is to be used either prior to, during, or after treatment of the subject with the other cancer therapy or, in the case of multiple combination therapies, either prior to, during, or after treatment of the subject with the other cancer therapies.

15 In yet other embodiments, the present invention provides for the use of the monoclonal antibody CHIR-12.12 or CHIR-5.9, or antigen-binding fragment thereof, in the manufacture of a medicament for treating CLL in a subject, wherein the medicament is coordinated with treatment with at least one other small molecule-based cancer therapy selected from the group consisting of SDX-101 (R-etodolac, 20 inducing apoptosis of malignant lymphocytes), second-generation purine nucleoside analogs such as clofarabine, inhibitors of production of the protein Bcl-2 by cancer cells (for example, the antisense agents oblimersen and Genasense®), proteasome inhibitors (for example, Velcade™ (bortezomib)), small molecule inhibitors of histone deacetylases (for example, hybrid/polar cytodifferentiation HPC) agents such 25 as suberanilohydroxamic acid (SAHA), and FR-901228), and any combinations thereof; or with other cancer therapy, for example, CD154 gene immunization (for example, ISF-154); wherein the medicament is to be used either prior to, during, or after treatment of the subject with the other cancer therapy or, in the case of multiple combination therapies, either prior to, during, or after treatment of the subject with the 30 other cancer therapies.

In some embodiments, the medicament comprising the antagonist anti-CD40 antibody, for example, the monoclonal antibody CHIR-12.12 or CHIR-5.9 disclosed

herein, or antigen-binding fragment thereof is coordinated with treatment with two other cancer therapies. Without being limiting, examples include coordination of the medicament with treatment with two chemotherapeutic agents, for example, coordination with treatment with fludarabine and cyclophosphamide; and

5 coordination of the medicament with treatment with a chemotherapeutic agent, for example, fludarabine, and another anti-cancer monoclonal antibody, for example, alemtuzumab, rituximab or other anti-CD20 antibody including the fully human antibody HuMax-CD20, R-1594, IMMU-106, TRU-015, AME-133, tositumomab/I-131 tositumomab (Bexxar®), and ibritumomab tiuxetan (Zevalin®), or anti-CD23

10 antibody. Where the medicament comprising the antagonist anti-CD40 antibody is coordinated with two other cancer therapies, use of the medicament can be prior to, during, or after treatment of the subject with either or both of the other cancer therapies.

The invention also provides for the use of an antagonist anti-CD40 antibody,

15 for example, the monoclonal antibody CHIR-12.12 or CHIR-5.9 disclosed herein, or antigen-binding fragment thereof in the manufacture of a medicament for treating CLL in a subject, wherein the medicament is used in a subject that has been pretreated with at least one other cancer therapy. By "pretreated" or "pretreatment" is intended the subject has been treated with one or more other cancer therapies prior to receiving

20 the medicament comprising the antagonist anti-CD40 antibody or antigen-binding fragment thereof. "Pretreated" or "pretreatment" includes subjects that have been treated with the other cancer therapy, or other cancer therapies, within 2 years, within 18 months, within 1 year, within 6 months, within 2 months, within 6 weeks, within 1 month, within 4 weeks, within 3 weeks, within 2 weeks, within 1 week, within 6 days,

25 within 5 days, within 4 days, within 3 days, within 2 days, or even within 1 day prior to initiation of treatment with the medicament comprising the antagonist anti-CD40 antibody, for example, the monoclonal antibody CHIR-12.12 or CHIR-5.9 disclosed herein, or antigen-binding fragment thereof. It is not necessary that the subject was a responder to pretreatment with the prior cancer therapy, or prior cancer therapies.

30 Thus, the subject that receives the medicament comprising the antagonist anti-CD40 antibody or antigen-binding fragment thereof could have responded, or could have failed to respond, to pretreatment with the prior cancer therapy, or to one or more of

the prior cancer therapies where pretreatment comprised multiple cancer therapies, for example, surgery and chemotherapy; surgery and other anti-cancer antibody therapy; chemotherapy and other anti-cancer antibody therapy; or surgery, chemotherapy, and other anti-cancer antibody therapy.

5 Thus, in some embodiments, the invention provides for the use of an antagonist anti-CD40 antibody, for example the monoclonal antibody CHIR-12.12 or CHIR-5.9 disclosed herein, or antigen-binding fragment thereof in the manufacture of a medicament that is used in a subject in need of treatment for CLL, where the subject has been pretreated with one or more of the following other cancer therapies: surgery;

10 radiation therapy; chemotherapy, optionally in combination with autologous bone marrow transplant, where suitable chemotherapeutic agents include, but are not limited to, fludarabine, chlorambucil, cladribine, vincristine, pentostatin, 2-chlorodeoxyadenosine, cyclophosphamide, doxorubicin, prednisone, and combinations thereof, for example, anthracycline-containing regimens such as CAP (cyclophosphamide, doxorubicin plus prednisone) and CHOP (cyclophosphamide, vincristine, prednisone plus doxorubicin); other anti-cancer monoclonal antibody therapy (for example, alemtuzumab (Campath[®]); rituximab (Rituxan[®]) or any other therapeutic anti-CD20 antibody; or anti-CD23 antibody targeting the CD23 antigen on malignant B cells); interferon-alpha therapy; interleukin-2 (IL-2) therapy; therapy

15 with IL-12, IL-15, or IL-21; or steroid therapy.

“Treatment” in the context of coordinated use of a medicament described herein with one or more other cancer therapies is herein defined as the application or administration of the medicament or of the other cancer therapy to a subject, or application or administration of the medicament or other cancer therapy to an isolated tissue or cell line from a subject, where the subject has chronic lymphocytic leukemia, a symptom associated with such a cancer, or a predisposition toward development of such a cancer, where the purpose is to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the cancer, any associated symptoms of the cancer, or the predisposition toward the development of the cancer.

30 The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Introduction

The antagonist anti-CD40 antibodies used in the examples below are CHIR-5.9 and CHIR-12.12. The CHIR-5.9 and CHIR-12.12 anti-CD40 antibodies are 5 human IgG₁ subtype anti-human CD40 monoclonal antibodies (mAbs) generated by immunization of transgenic mice bearing the human IgG₁ heavy chain locus and the human κ chain locus (XenoMouse® technology; Abgenix; Fremont, California). SF9 insect cells expressing CD40 extracellular domain were used as immunogen.

Briefly, splenocytes from immunized mice were fused with SP 2/0 or P 3 x 10 63Ag8.653 murine myeloma cells at a ratio of 10:1 using 50% polyethylene glycol as previously described by de Boer *et al.* (1988) *J. Immunol. Meth.* 113:143. The fused cells were resuspended in complete IMDM medium supplemented with hypoxanthine (0.1 mM), aminopterin (0.01 mM), thymidine (0.016 mM), and 0.5 ng/ml hIL-6 (Genzyme, Cambridge, Massachusetts). The fused cells were then distributed 15 between the wells of 96-well tissue culture plates, so that each well contained 1 growing hybridoma on average.

After 10-14 days, the supernatants of the hybridoma populations were 20 screened for specific antibody production. For the screening of specific antibody production by the hybridoma clones, the supernatants from each well were pooled and tested for anti-CD40 activity specificity by ELISA first. The positives were then used for fluorescent cell staining of EBV-transformed B cells using a standard FACS assay. Positive hybridoma cells were cloned twice by limiting dilution in IMDM/FBS containing 0.5 ng/ml hIL-6.

A total of 31 mice spleens were fused with the mouse myeloma SP2/0 cells to 25 generate 895 antibodies that recognize recombinant CD40 in ELISA. On average approximately 10% of hybridomas using Abgenix XenoMouse® technology (Abgenix; Fremont, California) may contain mouse lambda light chain instead of human kappa chain. The antibodies containing mouse light lambda chain were selected out. A subset of 260 antibodies that also showed binding to cell-surface 30 CD40 were selected for further analysis. Stable hybridomas selected during a series of subcloning procedures were used for further characterization in binding and functional assays. For further details of the selection process, see copending

provisional applications both entitled "*Antagonist Anti-CD40 Monoclonal Antibodies and Methods for Their Use*," filed November 4, 2003, and November 26, 2003, and assigned U.S. Patent Application Nos. 60/517,337 (Attorney Docket No. PP20107.001 (035784/258442)), and 60/525,579 (Attorney Docket No. PP20107.002 (035784/271525)), respectively; the contents of both of which are herein incorporated by reference in their entirety.

Clones from 7 other hybridomas were identified as having antagonistic activity. Based on their relative antagonistic potency and ADCC activities, two hybridoma clones were selected for further evaluation (Table 1 below). They are 10 named 131.2F8.5.9 (5.9) and 153.8E2.D10.D6.12.12 (12.12).

Table 1. Summary of initial set of data with anti-CD40 IgG1 antibodies CHIR-5.9 and CHIR-12.12.

Mother Hybridoma	Hybridoma clones	cell surface binding	Antagonist	ADCC	CDC	CMCC#	V-region DNA sequence
131.2F5	131.2F8.5.9	+++	+++	++	-	12047	Yes
153.8E2	153.8E2.D10.D6.12.12	+++	+++	++++	-	12056	Yes

15

Mouse hybridoma line 131.2F8.5.9 (CMCC#12047) and hybridoma line 153.8E2.D10.D6.12.12 (CMCC#12056) have been deposited with the American Type Culture Collection (ATCC; 10801 University Blvd., Manassas, Virginia 20110-2209 (USA)) under Patent Deposit Number PTA-5542 and PTA-5543, respectively.

20 The cDNAs encoding the variable regions of the candidate antibodies were amplified by PCR, cloned, and sequenced. The amino acid sequences for the light chain and heavy chain of the CHIR-12.12 antibody are set forth in Figures 1A and 1B, respectively. See also SEQ ID NO:2 (light chain for mAb CHIR-12.12) and SEQ ID NO:4 (heavy chain for mAb CHIR-12.12). A variant of the heavy chain for mAb 25 CHIR-12.12 is shown in Figure 1B (see also SEQ ID NO:5), which differs from SEQ ID NO:4 in having a serine residue substituted for the alanine residue at position 153 of SEQ ID NO:4. The nucleotide sequences encoding the light chain and heavy chain of the CHIR-12.12 antibody are set forth in Figures 2A and 2B, respectively. See also SEQ ID NO:1 (coding sequence for light chain for mAb CHIR-12.12) and SEQ ID 30 NO:3 (coding sequence for heavy chain for mAb CHIR-12.12). The amino acid

sequences for the light chain and heavy chain of the CHIR-5.9 antibody are set forth in Figures 3A and 3B, respectively. See also SEQ ID NO:6 (light chain for mAb CHIR-5.9) and SEQ ID NO:7 (heavy chain for mAb CHIR-5.9). A variant of the heavy chain for mAb CHIR-5.9 is shown in Figure 3B (see also SEQ ID NO:8),
5 which differs from SEQ ID NO:7 in having a serine residue substituted for the alanine residue at position 158 of SEQ ID NO:7.

As expected for antibodies derived from independent hybridomas, there is substantial variation in the nucleotide sequences in the complementarity determining regions (CDRs). The diversity in the CDR3 region of V_H is believed to most
10 significantly determine antibody specificity.

As shown by FACS analysis, CHIR-5.9 and CHIR-12.12 bind specifically to human CD40 and can prevent CD40-ligand binding. Both mAbs can compete off CD40-ligand pre-bound to cell surface CD40. The binding affinity of CHIR-5.9 to human CD40 is 1.2×10^{-8} M and the binding affinity of CHIR-12.12 to human CD40 is
15 5×10^{-10} M.

The CHIR-12.12 and CHIR-5.9 monoclonal antibodies are strong antagonists and inhibit *in vitro* CD40 ligand-mediated proliferation of normal B cells, as well as inhibiting *in vitro* CD40 ligand-mediated proliferation of cancer cells from NHL and CLL patients. *In vitro*, both antibodies kill primary cancer cells from NHL patients
20 by ADCC. Dose-dependent anti-tumor activity was seen in a xenograft human lymphoma model. For a more detailed description of these results, and the assays used to obtain them, see copending provisional applications both entitled "*Antagonist Anti-CD40 Monoclonal Antibodies and Methods for Their Use*," filed November 4, 2003, and November 26, 2003, and assigned U.S. Patent Application Nos. 60/517,337
25 (Attorney Docket No. PP20107.001 (035784/258442)), and 60/525,579 (Attorney Docket No. PP20107.002 (035784/271525)), respectively; the contents of both of which are herein incorporated by reference in their entirety.

B-cell chronic lymphocytic leukemia (CLL) is characterized by *in vivo* accumulation of long-lived CD5⁺ B cells. However, when cultured *in vitro*, CLL cells
30 die quickly by apoptosis. Protection from apoptosis *in vivo* is believed to result from supply of survival signals from the microenvironment. CD40 stimulation of CLL cells by CD40-ligand is identified to be one such survival signal.

The following studies were undertaken to determine if antagonist anti-CD40 mAbs CHIR-5.9 and CHIR-12.12 exhibit the following properties: (1) bind to chronic lymphocytic leukemia (CLL) patient cells; (2) promote cell death in CLL patient cells by blocking CD40-ligand induced survival signals; (3) have any 5 stimulatory/inhibitory activity by themselves for chronic lymphocytic leukemia (CLL) cells; and/or (4) mediate ADCC as a mode of action.

Example 1: CHIR-5.9 and CHIR-12.12 Can Block CD40-Mediated Survival and Proliferation of Cancer Cells from CLL Patients

10 The candidate antibodies can block CD40-mediated survival and proliferation of cancer cells from CLL patients. CLL cells from patients were cultured in suspension over CD40L-expressing formaldehyde-fixed CHO cells under two different conditions: addition of human isotype antibody IgG (control); and addition of either CHIR-5.9 or CHIR-12.12 monoclonal antibody. All antibodies were added 15 at concentrations of 1, 10, and 100 µg/mL in the absence of IL-4. The cell counts were performed at 24 and 48 h by MTS assay. Reduced numbers of cells were recovered from CHIR-5.9- (n=6) and CHIR-12.12- (n=2) treated cultures compared to control group. The greater differences in cell numbers between anti-CD40 mAb-treated and control antibody-treated cultures were seen at the 48-h time point. These data are 20 summarized in Table 2.

Table 2. The effect of candidate antibodies on CD40-induced survival and proliferation of cancer cells from CLL patients measured at 48h after the culture initiation

Patient#	Ab conc(µg/ml)	Relative cell numbers			% reduction in cell numbers*	
		IgG1	CHIR-5.9/5.11	CHIR-12.12	CHIR-5.9/5.11	CHIR-12.12
1	1	269.31	25.27	ND	90.62	ND
	10	101.58	33.07	ND	67.44	ND
	100	130.71	40.16	ND	69.28	ND
2	1	265.55	75.8	ND	71.46	ND
	10	227.57	128.5	ND	43.53	ND
	100	265.99	6.4	ND	97.59	ND
3	1	85.9	35.39	ND	58.80	ND
	10	70.44	39.51	ND	43.91	ND
	100	77.65	20.95	ND	73.02	ND
4	1	80.48	15.03	ND	81.32	ND
	10	63.01	19.51	ND	69.04	ND
	100	55.69	3.65	ND	93.45	ND
5	1	90.63	91.66	89.59	-1.14	1.15
	10	78.13	82.28	60.41	-5.31	22.68
	100	63.53	86.47	39.59	-36.11	37.68
6	1	130.21	77.6	71.88	40.40	44.80
	10	131.77	78.13	73.96	40.71	43.87
	100	127.08	76.56	82.29	39.75	35.25

* % reduction compared to control Abs=100-(test Abs/control Abs)*100

5 A second study revealed similar results. In this study, primary CLL cells from
 9 patients were cultured in suspension over CD40L-expressing formaldehyde-fixed
 CHO cells in the presence or absence of 1, 10, or 100 µg/ml anti-CD40 mAb CHIR-
 12.12 in a manner described above, using non-specific IgG as the control. After 48
 and 72 hours, proliferation of the cultures was measured as noted above. In the
 10 absence of anti-CD40 mAb CHIR-12.12, the primary CLL cells either resisted
 spontaneous cell death or proliferated. This effect was inhibited in the presence of
 mAb CHIR-12.12, which restored CLL cell death. Thus, these results demonstrate
 inhibition of CD40-induced CLL cell proliferation by the anti-CD40 mAb CHIR-
 12.12 at both 48 and 72 hours. See Figures 5A and 5B.

15 Similar experiments were performed on unstimulated CLL cells in the
 presence of mAb CHIR-12.12 alone. The mAb CHIR-12.12 (10 µg/ml) alone did not

induce CLL proliferation and thus did not have a stimulatory effect on CLL cells as compared to control IgG (10 µg /ml) at 48 and 72 hours. See Figures 6A and 6B.

Example 2: Ability of Anti-CD40 mAb CHIR-12.12 to Lyse Chronic Lymphocytic Leukemia (CLL) Cell Lines by Antibody-Dependent Cellular Cytotoxicity (ADCC)

The CLL cell line EHEB was cultured with antagonist anti-CD40 mAb CHIR-12.12 or the anti-CD20 antibody Rituxan® (IDEC Pharmaceuticals Corp., San Diego, California) and freshly isolated human NK cells from normal volunteer blood donors 10 as effector cells. The percent specific lysis was measured based on the release of marker from target cells.

The anti-CD40 mAb CHIR-12.12 showed lysis activity in a dose-dependent manner and reached maximum lysis levels at 0.1 µg/ml (Figure 7). As shown in Figure 7, mAb CHIR-12.12 induced greater ADCC-mediated cell lysis than Rituxan® 15 (maximum specific lysis with mAb CHIR-12.12 = 27.2% versus maximum specific lysis with Rituxan® = 16.2%; p=0.007). Based on these results, approximately 10-fold more binding sites for anti-CD20 antibody than for anti-CD40 antibody were present on the target cells (see Table 3), indicative of fewer CD40 molecules being expressed on CLL cell line EHEB when compared to CD20 expression. In fact, the CLL target 20 cell line expressed 509,053±13,560 CD20 molecules compared to 48,416±584 CD40 molecules. Thus, the greater ADCC mediated by mAb CHIR-12.12 was not due to higher density of CD40 molecules on this CLL cell line compared to CD20 molecules.

25 Table 3: EHEB cell line target binding site ratio.

Cell Line	% Maximum Lysis		Ratio of Maximum Binding Sites CD20/CD40
	mAb CHIR-12.12	Rituxan®	
EHEB	27.19	16.21	10.51

Example 3: CHIR-5.9 and CHIR-12.12 Bind to a Different Epitope on CD40 than 15B8

The candidate monoclonal antibodies CHIR-5.9 and CHIR-12.12 compete with each other for binding to CD40 but not with 15B8, an IgG₂ anti-CD40 mAb (see 5 International Publication No. WO 02/28904). Antibody competition binding studies using Biacore were designed using CM5 biosensor chips with protein A immobilized via amine coupling, which was used to capture either anti-CD40, CHIR-12.12, or 15B8. Normal association/dissociation binding curves are observed with varying concentrations of CD40-his (data not shown). For competition studies, either CHIR- 10 12.12 or 15B8 were captured onto the protein A surface. Subsequently a CD40-his / CHIR-5.9 Fab complex (100 nM CD40:1 μM CHIR-5.9 Fab), at varying concentrations, was flowed across the modified surface. In the case of CHIR-12.12, there was no association of the complex observed, indicating CHIR-5.9 blocks 15 binding of CHIR-12.12 to CD40-his. For 15B8, association of the Fab CHIR-5.9 complex was observed indicating CHIR-5.9 does not block binding of 15B8 to CD40 binding site. However, the off rate of the complex dramatically increased (data not shown).

It has also been determined that 15B8 and CHIR-12.12 do not compete for 20 CD40-his binding. This experiment was set up by capturing CHIR-12.12 on the protein A biosensor chip, blocking residual protein A sites with control hIgG₁, binding CD40-his and then flowing 15B8 over the modified surface. 15B8 did bind under these conditions indicating CHIR-12.12 does not block 15B8 from binding to CD40.

25 Example 4: Binding Properties of CHIR-12.12 and CHIR-5.9 mAB

Protein A was immobilized onto CM5 biosensor chips via amine coupling. Human anti-CD40 monoclonal antibodies, at 1.5 μg/ml, were captured onto the 30 modified biosensor surface for 1.5 minutes at 10 μl/min. Recombinant soluble CD40-his was flowed over the biosensor surface at varying concentrations. Antibody and antigen were diluted in 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20 (HBS-EP). Kinetic and affinity constants were determined using the Biaevaluation software with a 1:1 interaction model/global fit.

As shown in Table 4 below, there is 121-fold difference in the off rate of CHIR-5.9 and CHIR-12.12 resulting in 24-fold higher affinity for CHIR-12.12.

5 Table 4. Summary of binding properties of CHIR-5.9 and CHIR-12.12 anti-CD40 antibodies.

Antibody	K_a (M ⁻¹ sec ⁻¹)	k_d (sec ⁻¹)	K_D (nM)
Anti-CD40, CHIR-5.9	(12.35 ± 0.64) x 10 ⁵	(15.0 ± 1.3) x 10 ⁻³	12.15 ± 0.35
Anti-CD40, CHIR-12.12	(2.41 ± 0.13) x 10 ⁵	(1.24 ± 0.06) x 10 ⁻⁴	0.51 ± 0.02

10 To determine the location of the epitope on CD40 recognized by monoclonal antibodies CHIR-12.12 and CHIR-5.9, SDS-PAGE and Western blot analysis were performed. Purified CD40 (0.5 µg) was separated on a 4-12% NUPAGE gel under reducing and non-reducing conditions, transferred to PVDF membranes, and probed with monoclonal antibodies at 10 µg/ml concentration. Blots were probed with alkaline phosphatase conjugated anti-human IgG and developed using the Western 15 Blue^R stabilized substrate for alkaline phosphatase (Promega).

Results indicate that anti-CD40 monoclonal antibody CHIR-12.12 recognizes epitopes on both the non-reduced and reduced forms of CD40, with the non-reduced form of CD40 exhibiting greater intensity than the reduced form of CD40 (Table 5; blots not shown). The fact that recognition was positive for both forms of CD40 20 indicates that this antibody interacts with a conformational epitope part of which is a linear sequence. Monoclonal antibody CHIR-5.9 primarily recognizes the non-reduced form of CD40 suggesting that this antibody interacts with a primarily conformational epitope (Table 5; blots not shown).

Table 5. Domain identification.

	Domain 1	Domain 2	Domain 3	Domain 4
mAb CHIR-12.12	-	+	-	-
mAb CHIR-5.9	-	+	-	-
mAb 15B8	+	-	-	-

To map the antigenic region on CD40, the four extracellular domains of CD40
 5 were cloned and expressed in insect cells as GST fusion proteins. The secretion of the
 four domains was ensured with a GP67 secretion signal. Insect cell supernatant was
 analyzed by SDS-PAGE and western blot analysis to identify the domain containing
 the epitope.

Monoclonal antibody CHIR-12.12 recognizes an epitope on Domain 2 under
 10 both reducing and non-reducing conditions (Table 6; blots not shown). In contrast,
 monoclonal antibody CHIR-5.9 exhibits very weak recognition to Domain 2 (Table 6;
 blots not shown). Neither of these antibodies recognizes Domains 1, 3, or 4 in this
 analysis.

15 Table 6. Domain 2 analysis.

	Reduced	Non-reduced
mAb CHIR-12.12	++	+++
mAb CHIR-5.9	+	+

To define more precisely the epitope recognized by mAb CHIR-12.12,
 peptides were synthesized from the extracellular Domain 2 of CD40, which
 corresponds to the sequence

20 PCGESEFLDTWNRETHCHQHKYCDPNLGLRVQQKGTSETDTICT (residues 61-
 104 of the sequence shown in SEQ ID NO:10 or SEQ ID NO:12). SPOTS membranes
 (Sigma) containing thirty-five 10mer peptides with a 1-amino-acid offset were
 generated. Western blot analysis with mAb CHIR-12.12 and anti-human IgG beta-
 galactosidase as secondary antibody was performed. The blot was stripped and
 25 reprobed with mAb CHIR-5.9 to determine the region recognized by this antibody

SPOTs analysis probing with anti-CD40 monoclonal antibody CHIR-12.12 at 10 µg/ml yielded positive reactions with spots 18 through 22. The sequence region covered by these peptides is shown in Table 7.

5 Table 7. Results of SPOTs analysis probing with anti-CD40 monoclonal antibody CHIR-12.12.

Spot Number	Sequence Region
18	HQHKYCDPNL (residues 78-87 of SEQ ID NO:10 or 12)
19	QHKYCDPNLG (residues 79-88 of SEQ ID NO:10 or 12)
20	HKYCDPNLGL (residues 80-89 of SEQ ID NO:10 or 12)
21	KYCDPNLGLR (residues 81-90 of SEQ ID NO:10 or 12)
22	YCDPNLGLRV (residues 82-91 of SEQ ID NO:10 or 12)

10 These results correspond to a linear epitope of: YCDPNL (residues 82-87 of the sequence shown in SEQ ID NO:10 or SEQ ID NO:12). This epitope contains Y82, D84, and N86, which have been predicted to be involved in the CD40-CD40 ligand interaction.

15 SPOTs analysis with mAb CHIR-5.9 showed a weak recognition of peptides represented by spots 20-22 shown in Table 8, suggesting involvement of the region YCDPNLGL (residues 82-89 of the sequence shown in SEQ ID NO:10 or SEQ ID NO:12) in its binding to CD40. It should be noted that the mAbs CHIR-12.12 and CHIR-5.9 compete with each other for binding to CD40 in BIACORE analysis.

20 Table 8. Results of SPOTs analysis probing with anti-CD40 monoclonal antibody CHIR-5.9.

Spot Number	Sequence Region
20	HKYCDPNLGL (residues 80-89 of SEQ ID NO:10 or 12)
21	KYCDPNLGLR (residues 81-90 of SEQ ID NO:10 or 12)
22	YCDPNLGLRV (residues 82-91 of SEQ ID NO:10 or 12)

The linear epitopes identified by the SPOTs analyses are within the CD40 B1 module. The sequence of the CD40 B1 module is:

HKYCDPNLGLRVQQKGTSETDTIC (residues 80-103 of SEQ ID NO:10 or 12).

Within the linear epitope identified for CHIR-12.12 is C83. It is known that
5 this cysteine residue forms a disulphide bond with C103. It is likely that the conformational epitope of the CHIR-12.12 mAb contains this disulfide bond (C83-C103) and/or surrounding amino acids conformationally close to C103.

Example 6: CHIR-12.12 Blocks CD40L-Mediated CD40 Survival and Signaling

10 Pathways in Normal Human B Cells

Soluble CD40 ligand (CD40L) activates B cells and induces various aspects of functional responses, including enhancement of survival and proliferation, and activation of NF κ B, ERK/MAPK, PI3K/Akt, and p38 signaling pathways. In addition, CD40L-mediated CD40 stimulation provides survival signals by reduction
15 of cleaved PARP and induction of the anti-apoptotic proteins, XIAP and Mcl-1, in normal B cells. CD40L-mediated CD40 stimulation also recruits TRAF2 and TRAF3 to bind CD40 cytoplasmic domain.

The following studies demonstrate that CHIR-12.12 directly inhibited all of these stimulation effects on normal human B cells. For example, CHIR-12.12
20 treatment resulted in increased cleavage of caspase-9, caspase-3, and PARP as well as reduction of XIAP and Mcl-1 in a time- and dose-dependent manner, restoring B cell apoptosis. Treatment with CHIR-12.12 also inhibited phosphorylation of I κ B kinase (IKK) α and β (NF κ B pathway), ERK, Akt, and p38 in response to CD40L-mediated CD40 stimulation. Further, it was found that CHIR-12.12 did not trigger these
25 apoptotic effects without initial CD40L-mediated CD40 stimulation.

CHIR-12.12 inhibited survival mediated by CD40 ligand by inducing cleavage of PARP.

In these experiments, 0.6×10^6 normal human B cells from healthy donors
30 (percent purity between 85-95%) were stimulated with 1 μ g/ml sCD40L (Alexis Corp., Bingham, Nottinghamshire, UK). CHIR-12.12 (10 μ g/ml) and control IgG were then added. Cells were collected at 0, 20 minutes, 2 hours, 6 hours, 18 hours,

and 26 hours. Cleaved caspase-9, cleaved caspase-3, cleaved PARP, and β -actin controls were detected in cell lysates by Western blot.

Briefly, it was observed that CD40L-mediated CD40 stimulation provided survival signals as it did not result in increases of cleaved caspase-9, cleaved caspase-
5 3, or cleaved PARP over time, indicating that the cells were not undergoing apoptosis. However, treatment with CHIR-12.12 resulted in an increase of these cleavage products, indicating that CHIR-12.12 treatment abrogated the effects of CD40L binding on survival signaling in sCD40L-stimulated normal B cells, restoring B cell apoptosis (data not shown).

10

CHIR-12.12 inhibited expression of "survival" anti-apoptotic proteins.

In these experiments, 0.6×10^6 normal human B cells from healthy donors (percent purity between 85-95%) were stimulated with 1 $\mu\text{g}/\text{ml}$ sCD40L (Alexis Corp., Bingham, Nottinghamshire, UK). CHIR-12.12 (10 $\mu\text{g}/\text{ml}$) and control IgG
15 were then added. Cells were collected at 0, 20 minutes, 2 hours, 6 hours, 18 hours, and 26 hours. Mcl-1, XIAP, CD40, and β -actin controls were detected in cell lysates by Western blot. Briefly, sCD40L stimulation resulted in sustained expression of Mcl-1 and XIAP over time. However, treatment of the sCD40L-stimulated cells with CHIR 12.12 resulted in a decrease in expression of these proteins overtime (data not
20 shown). Since Mcl-1 and XIAP are "survival" signals capable of blocking the apoptotic pathway, these results demonstrate that CHIR-12.12 treatment removes the blockade against apoptosis in sCD40L-stimulated normal B cells.

25 *CHIR-12.12 treatment inhibited phosphorylation of IKK α (Ser180) and IKK β (Ser 181) in normal B cells.*

In these experiments, 1.0×10^6 normal human B cells from healthy donors (percent purity between 85-95%) were stimulated with 1 $\mu\text{g}/\text{ml}$ sCD40L (Alexis Corp., Bingham, Nottinghamshire, UK). CHIR-12.12 (10 $\mu\text{g}/\text{ml}$) and control IgG were then added. Cells were collected at 0 and 20 minutes. Phosphorylated IKK α

(Ser180) and IKK β (Ser 181) and total IKK β controls were detected in cell lysates by Western blot.

Briefly, stimulation by sCD40L resulted in phosphorylation of IKK α (Ser180) and IKK β (Ser 181) over time; however, treatment with CHIR-12.12 abrogated this 5 response to sCD40L stimulation in normal B cells (data not shown).

CHIR-12.12 treatment inhibited survival mediated by CD40 ligand in a dose-dependent manner.

In these experiments, 0.6×10^6 normal human B cells from healthy donors 10 percent purity between 85-95%) were stimulated with 1 $\mu\text{g}/\text{ml}$ sCD40L (Alexis Corp., Bingham, Nottinghamshire, UK). CHIR-12.12 (0.01, 0.1, 0.2, 0.5, 1.0 $\mu\text{g}/\text{ml}$) and control IgG were then added. Cells were collected at 24 hours. Cleaved PARP, and β -actin controls were detected in cell lysates by Western blot.

Briefly, CHIR-12.12 treatment resulted in increase of PARP cleavage in 15 sCD40L stimulated cells in a dose-dependent manner and therefore abrogated the survival signaling pathway in sCD40L-stimulated normal B cells (data not shown).

CHIR-12.12 inhibited expression of "survival" anti-apoptotic proteins in a dose-dependent manner.

20 In these experiments, 0.6×10^6 normal human B cells from healthy donors (percent purity between 85-95%) were stimulated with 1 $\mu\text{g}/\text{ml}$ sCD40L (Alexis Corp., Bingham, Nottinghamshire, UK). CHIR-12.12 (0.5, 2, and 10 $\mu\text{g}/\text{ml}$) and control IgG were then added. Cells were collected at 22 hours. Mcl-1, XIAP, cleaved PARP, and β -actin controls were detected in cell lysates by Western blot.

25 Briefly, CHIR-12.12 treatment reduced Mcl-1 and XIAP expression and increased cleaved PARP expression in sCD40L-stimulated cells in a dose-dependent manner, and thus abrogated these blockades to the apoptotic pathway in sCD40L-stimulated normal B cells (data not shown).

CHIR-12.12 did not affect expression of anti-apoptotic proteins, cleaved-PARP, and XIAP, in the absence of soluble CD40L signaling.

In these experiments, 1.0×10^6 normal human B cells from healthy donors (percent purity between 85-95%) were treated with CHIR-12.12 (10 $\mu\text{g/ml}$) and 5 control IgG only (i.e., cells were not pre-stimulated with sCD40L before adding antibody). Cells were collected at 0, 4, 14, and 16 hours. XIAP, cleaved PARP, and β -actin controls were detected in cell lysates by Western blot.

Briefly, the results show that without sCD40L stimulation, the cells expressed increased concentrations of cleaved PARP, while expression of XIAP remained 10 constant, in both IgG treated control cells and CHIR-12.12 cells (data not shown). These data indicate that CHIR-12.12 does not trigger apoptosis in normal human B cells without CD40L stimulation.

15 *CHIR-12.12 inhibits phosphorylation of IKK α (Ser180) and IKK β (Ser181), Akt, ERK, and p38 in normal B cells.*

In these experiments, 1.0×10^6 normal human B cells from healthy donors (percent purity between 85-95%) were serum starved in 1% FBS-containing media and stimulated with 1 $\mu\text{g/ml}$ sCD40L (Alexis Corp., Bingham, Nottinghamshire, UK). The cultures were treated with CHIR-12.12 (1 and 10 $\mu\text{g/ml}$) and control IgG. Cells 20 were collected at 0 and 20 minutes. Phospho-IKK α , phospho-IKK β , total IKK β , phospho-ERK, total ERK, phospho-Akt, total Akt, phospho-p38, and total p38 were detected in cell lysates by Western blot.

Briefly, sCD40L stimulation resulted in increases in IKK α/β phosphorylation, 25 ERK phosphorylation, Akt phosphorylation, and p38 phosphorylation, thus leading to survival and or proliferation of the cells. Treatment of the cells with CHIR-12.12 abrogated the effects of sCD40L stimulation on these signaling pathways in normal B cells (data not shown).

30 *CHIR 12.12 inhibits multiple signaling pathways such as PI3K and MEK /ERK in the CD40 signaling cascade.*

In these experiments, 1.0×10^6 normal human B cells from healthy donors (percent purity between 85-95%) were serum starved in 1% FBS-containing media and stimulated with 1 $\mu\text{g}/\text{ml}$ sCD40L (Alexis Corp., Bingham, Nottinghamshire, UK). The cultures were also treated with CHIR-12.12 (1 and 10 $\mu\text{g}/\text{ml}$), Wortmanin, (a 5 PI3K/Akt inhibitor; 1 and 10 μM), LY 294002 (a PI3K/Akt inhibitor; 10 and 30 μM), and PD 98095 (a MEK inhibitor; 10 and 30 $\mu\text{g}/\text{ml}$). Cells were collected at 0 and 20 minutes. Phospho-ERK, phospho-Akt, total Akt, phospho-IKK α/β , and total were detected in cell lysates by Western blot.

Briefly, the results show that CHIR-12.12 abrogated the phosphorylation of all 10 of these signal transduction molecules, whereas the signal transduction inhibitors showed only specific abrogation of signaling, indicating that CHIR-12.12 likely inhibits upstream of these signal transduction molecules mediated by CD40L stimulation (data not shown).

15 *CHIR-12.12 inhibits the binding of signaling molecules TRAF2 and TRAF3 to the cytoplasmic domain of CD40 in normal B cells.*

In these experiments, 4.0×10^6 normal human B cells from healthy donors (percent purity between 85-95%) were serum starved for four hours in 1% FBS-containing media and stimulated with 1 $\mu\text{g}/\text{ml}$ sCD40L (Alexis Corp., Bingham, 20 Nottinghamshire, UK) for 20 minutes. Cells were collected at 0 and 20 minutes. CD40 was immunoprecipitated using polyclonal anti-CD40 (Santa Cruz Biotechnology, CA), and was probed in a Western blot with anti-TRAF2 mAb (Santa Cruz Biotechnology, CA), anti-TRAF3 mAb (Santa Cruz Biotechnology, CA), and anti-CD40 mAb (Santa Cruz Biotechnology, CA).

25 Briefly, the results show that TRAF2 and TRAF3 co-precipitated with CD40 after sCD40L stimulation. In contrast, treatment with CHIR-12.12 abrogated formation of the CD40-TRAF2/3 signaling complex in sCD40L-stimulated normal B cells. There were no changes in CD40 expression (data not shown).

Without being bound by theory, the results of these experiments, and the 30 results in the examples outlined above, indicate that the CHIR-12.12 antibody is a dual action antagonist anti-CD40 monoclonal antibody having a unique combination of attributes. This fully human monoclonal antibody blocks CD40L-mediated CD40

signaling pathways for survival and proliferation of B cells; this antagonism leads to ultimate cell death. CHIR-12.12 also mediates recognition and binding by effector cells, initiating antibody dependent cellular cytotoxicity (ADCC). Once CHIR-12.12 is bound to effector cells, cytolytic enzymes are released, leading to B-cell apoptosis and lysis. CHIR-12.12 is a more potent anti-tumor antibody than is rituximab when compared in pre-clinical tumor models.

Example 7: Liquid Pharmaceutical Formulation for Antagonist Anti-CD40 Antibodies

10 The objective of this study was to investigate the effects of solution pH on stability of the antagonist anti-CD40 antibody CHIR-12.12 by both biophysical and biochemical methods in order to select the optimum solution environment for this antibody. Differential Scanning Calorimetry (DSC) results showed that the conformation stability of CHIR-12.12 is optimal in formulations having pH 5.5-6.5.

15 Based on a combination of SDS-PAGE, Size-Exclusion HPLC (SEC-HPLC), and Cation-Exchange HPLC (CEX-HPLC) analysis, the physicochemical stability of CHIR-12.12 is optimal at about pH 5.0-5.5. In view of these results, one recommended liquid pharmaceutical formulation comprising this antibody is a formulation comprising CHIR-12.12 at about 20 mg/ml formulated in about 10 mM sodium succinate, about 150 mM sodium chloride, and having a pH of about pH 5.5.

20

Materials and Methods

The CHIR-12.12 antibody used in the formulation studies is a human monoclonal antibody produced by a CHO cell culture process. This MAb has a molecular weight of 150 kDa and consists of two light chains and two heavy chains linked together by disulfide bands. It is targeted against the CD40 cell surface receptor on CD40-expressing cells, including normal and malignant B cells, for treatment of various cancers and autoimmune/inflammatory diseases.

30 The anti-CD40 drug substance used for this study was a CHO-derived purified anti-CD40 (CHIR-12.12) bulk lot. The composition of the drug substance was 9.7 mg/ml CHIR-12.12 antibody in 10 mM sodium citrate, 150 mM sodium chloride, at pH 6.5. The control sample in the study was the received drug substance, followed by

freezing at $\leq -60^{\circ}\text{C}$, thawing at RT and testing along with stability samples at predetermined time points. The stability samples were prepared by dialysis of the drug substance against different pH solutions and the CHIR-12.12 concentration in each sample was determined by UV 280 as presented in Table 9.

5

Table 9. CHIR-12.12 formulations.

Buffer Composition	pH	CHIR-12.12 Concentration (mg/ml)
10 mM sodium citrate, 150 mM sodium chloride	4.5	9.0
10 mM sodium succinate, 150 mM sodium chloride	5.0	9.3
10 mM sodium succinate, 150 mM sodium chloride	5.5	9.2
10 mM sodium citrate, 150 mM sodium chloride	6.0	9.7
10 mM sodium citrate, 150 mM sodium chloride	6.5	9.4
10 mM sodium phosphate, 150 mM sodium chloride	7.0	9.4
10 mM sodium phosphate, 150 mM sodium chloride	7.5	9.5
10 mM glycine, 150 mM sodium chloride	9.0	9.5

10 Physicochemical stability of the CHIR-12.12 antibody in the various formulations was assayed using the following protocols.

Differential Scanning Calorimetry (DSC)

15 Conformational stability of different formulation samples was monitored using a MicroCal VP-DSC upon heating 15°C to 90°C at $1^{\circ}\text{C}/\text{min}$.

SDS-PAGE

20 Fragmentation and aggregation were estimated using 4-20% Tris-Glycine Gel under non-reducing and reducing conditions. Protein was detected by Coomassie blue staining.

Size Exclusion Chromatograph (SEC-HPLC)

25 Protein fragmentation and aggregation were also measured by a Water Alliance HPLC with a Tosohas TSK-GEL 3000SWXL column, 100 mM sodium phosphate, pH 7.0 as mobile phase at a flow rate of 0.7 ml/min.

30 *Cation Exchange Chromatography (CEX-HPLC)*

Charge change related degradation was measured using Waters 600s HPLC system with a Dionex Propac WCX-10 column, 50 mM HEPES, pH 7.3 as mobile phase A and 50 mM HEPES containing 500 mM NaCl, pH 7.3 as mobile phase B at a flow rate of 0.5°C/min.

5

Results and Discussion

Conformational stability study.

10 Thermal unfolding of CHIR-12.12 revealed at least two thermal transitions, probably representing unfolding melting of the Fab and the Fc domains, respectively. At higher temperatures, the protein presumably aggregated, resulting in loss of DSC signal. For the formulation screening purpose, the lowest thermal transition temperature was defined as the melting temperature, T_m, in this study. Figure 8
15 shows the thermal melting temperature as a function of formulation pHs. Formulations at pH 5.5-6.5 provided anti-CD40 with higher conformational stability as demonstrated by the higher thermal melting temperatures.

20

SDS-PAGE analysis.

The CHIR-12.12 formulation samples at pH 4.5-9.0 were incubated at 40°C for 2 months and subjected to SDS-PAGE analysis (data not shown). Under non-reducing conditions, species with molecular weight (MW) of 23 kDa and 27 kDa were observed in formulations above pH 5.5, and species with MW of 51 kDa were observed in all formulations, but appeared less at pH 5.0-5.5. A species with MW of 100 kDa could be seen at pH 7.5 and pH 9.0.

Under reducing conditions, CHIR-12.12 was reduced into free heavy chains and light chains with MW of 50 kDa and 24 kDa, respectively. The 100 kDa species seemed not fully reducible and increased with increasing solution pH, suggesting non-disulfide covalent association might occur in the molecules. Since there were other species with unknown identities on SDS-PAGE, stability comparison of each formulation is based on the remaining purity of CHIR-12.12. Formulations at pH 5.0-35 6.0 provided a more stable environment to CHIR-12.12. Few aggregates were detected by SDS-PAGE (data not shown).

SEC-HPLC analysis .

5 SEC-HPLC analysis detected the intact CHIR-12.12 as the main peak species, an aggregation species as a front peak species separate from the main peak species, a large fragment species as a shoulder peak on the back of the main peak species, and small fragment species were detected post-main peak species. After incubation at 5°C and 25°C for 3 months, negligible amounts of protein fragments and aggregates

10 (<1.0%) were detected in the above formulations and the CHIR-12.12 main peak species remained greater than 99% purity (data not shown). However, protein fragments gradually developed upon storage at 40°C and more fragments formed at pH 4.5 and pH 6.5-9.0, as shown in Table 10. After incubating the CHIR-12.12 formulations at 40°C for 3 months, about 2-3% aggregates were detected in pH 7.5

15 and pH 9.0, while less than 1% aggregates were detected in other pH formulations (data not shown). The SEC-HPLC results indicate CHIR-12.12 is more stable at about pH 5.0-6.0.

20 Table 10. SEC-HPLC results of CHIR-12.12 stability samples under real-time and accelerated storage conditions.

Sample	Main peak %				Fragments %			
	t=0	40°C 1 m	40°C 2 m	40°C 3 m	t=0	40°C 1 m	40°C 2 m	40°C 3 m
Control	99.4	99.2	99.9	99.5	<1.0	<1.0	<1.0	<1.0
pH 4.5	99.4	93.2	86.0	81.3	<1.0	6.4	13.2	18.1
pH 5.0	99.8	98.7	91.3	89.2	<1.0	<1.0	7.8	10.2
pH 5.5	99.8	98.9	91.4	90.6	<1.0	<1.0	7.6	8.8
pH 6.0	99.6	97.7	90.4	87.3	<1.0	1.9	8.2	11.7
pH 6.5	99.3	93.4	89.0	86.9	<1.0	5.6	9.9	12.4
pH 7.0	99.2	93.9	87.4	85.1	<1.0	5.5	11.1	13.5
pH 7.5	99.1	92.8	84.4	81.9	<1.0	6.4	12.9	16.2
pH 9.0	99.3	82.4	61.6	50.6	<1.0	15.4	36.2	47.6

CEX-HPLC analysis.

CEX-HPLC analysis detected the intact CHIR-12.12 as the main peak species, acidic variants eluted earlier than the main peak species, and C-terminal lysine

addition variants eluted post-main peak species. Table 11 shows the dependence of the percentages of the remaining main peak CHIR-12.12 species and acidic variants on solution pH. The control sample already contained a high degree of acidic species (~33%), probably due to early-stage fermentation and purification processes. The 5 susceptibility of CHIR-12.12 to higher pH solutions is evidenced by two facts. First, the initial formulation sample at pH 9.0 (t=0) already generated 12% more acidic species than the control. Second, the percentage of acidic species increased sharply with increasing pH. The charge change-related degradation is likely due to deamidation. The above data indicate that this type of degradation of CHIR-12.12 was 10 minimized at about pH 5.0-5.5.

Table 11. Percentage of peak area by CEX-HPLC for CHIR-12.12 in different pH formulations under real-time and accelerated storage conditions.

Sample	Main peak %					Acidic variants %				
	t=0	5°C 3m	25°C 3 m	40°C 1 m	40°C 2 m	t=0	5°C 3m	25°C 3 m	40°C 1 m	40°C 2 m
Control	49.2	49.8	49.8	49.2	50.3	32.0	33.7	33.7	32.0	33.6
pH 4.5	48.5	49.7	43.7	39.7	30.0	32.5	32.6	38.0	44.2	56.4
pH 5.0	49.6	49.8	48.3	40.6	31.4	32.7	31.8	35.0	44.3	57.1
pH 5.5	50.7	50.3	48.1	40.0	30.2	32.6	31.8	37.8	48.9	63.3
pH 6.0	50.2	49.9	47.9	37.4	23.9	33.1	33.6	38.5	54.9	72.7
pH 6.5	49.4	49.9	42.3	29.7	14.6	33.3	33.6	47.7	65.2	84.6
pH 7.0	49.7	49.9	21.9	-	-	34.4	36.4	64.4	-	-
pH 7.5	49.3	48.3	12.7	-	-	35.5	40.1	79.2	-	-
pH 9.0	41.3	31.8	-	-	-	44.7	59.9	-	-	-

15

Conclusion

The pH has a significant effect on conformational and physicochemical stabilities of CHIR-12.12. Charge change-related degradation was determined to be 20 the main degradation pathway for CHIR-12.12, which was minimized at pH 5.0-5.5. Based on overall stability data, one recommended liquid pharmaceutical formulation comprising this antibody is a formulation comprising CHIR-12.12 at about 20 mg/ml formulated in about 10 mM sodium succinate, about 150 mM sodium chloride, and having a pH of about pH 5.5.

25

Example 8: Clinical Studies with CHIR-5.9 and CHIR-12.12***Clinical Objectives***

The overall objective is to provide an effective therapy for chronic lymphocytic leukemia (CLL) by targeting these cancer cells with an anti-CD40 IgG1. The signal for this disease is determined in phase I although some measure of activity may be obtained in phase I. Initially the agent is studied as a single agent, but will be combined with other agents, chemotherapeutics, and radiation therapy, as development proceeds.

10 Phase I

- Evaluate safety and pharmacokinetics – dose escalation in subjects with chronic lymphocytic leukemia (CLL).
- Choose dose based on safety, tolerability, and change in serum markers of CD40. In general an MTD is sought but other indications of efficacy (depletion of CD40⁺ CLL cells, etc.) may be adequate for dose finding.
- Consideration of more than one dose, as some dose finding may be necessary in phase II.
- Patients are dosed weekly with real-time pharmacokinetic (Pk) sampling. Initially a 4-week cycle is the maximum dosing allowed. The Pk may be highly variable depending on the disease state, density of CD40 etc.
- This trial(s) is open to subjects with CLL.
- Decision to discontinue or continue studies is based on safety, dose, and preliminary evidence of anti-tumor activity.
- Activity of drug as determined by response rate is determined in Phase II.
- Identify dose(s) for Phase II.

Phase II

Several trials will be initiated in subjects with CLL. More than one dose, and more than one schedule may be tested in a randomized phase II setting.

- Target a CLL population that has failed current standard of care
(chemotherapy failures)

5 ✓ Decision to discontinue or continue with study is based on proof of therapeutic concept in Phase II

✓ Determine whether surrogate marker can be used as early indication of clinical efficacy

✓ Identify doses for Phase III

10 **Phase III**

Phase III will depend on where the signal is detected in phase II, and what competing therapies are considered to be the standard. If the signal is in a stage of disease where there is no standard of therapy, then a single arm, well-controlled study could serve as a pivotal trial. If there are competing agents that are considered 15 standard, then head-to-head studies are conducted.

Many modifications and other embodiments of the inventions set forth herein will come to mind to one skilled in the art to which these inventions pertain having the benefit of the teachings presented in the foregoing descriptions and the associated 20 drawings. Therefore, it is to be understood that the inventions are not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims and list of embodiments disclosed herein. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

25 All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

30

THAT WHICH IS CLAIMED:

1. A method for treating a human subject for chronic lymphocytic leukemia (CLL), comprising administering to said subject an effective amount of a human anti-CD40 monoclonal antibody that is capable of specifically binding to a human CD40 antigen expressed on the surface of a human CD40-expressing cell, said monoclonal antibody being free of significant agonist activity, whereby, when said monoclonal antibody binds to the CD40 antigen expressed on the surface of said cell, the growth or differentiation of said cell is inhibited, said human anti-CD40 monoclonal antibody being selected from the group consisting of:
 - a) the monoclonal antibody CHIR-5.9 or CHIR-12.12;
 - b) the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;
 - c) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:6, the sequence shown in SEQ ID NO:7, the sequence shown in SEQ ID NO:8, both the sequence shown in SEQ ID NO:6 and SEQ ID NO:7, and both the sequence shown in SEQ ID NO:6 and SEQ ID NO:8;
 - d) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:2, the sequence shown in SEQ ID NO:4, the sequence shown in SEQ ID NO:5, both the sequence shown in SEQ ID NO:2 and SEQ ID NO:4, and both the sequence shown in SEQ ID NO:2 and SEQ ID NO:5;
 - e) a monoclonal antibody having an amino acid sequence encoded by a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of the sequence shown in SEQ ID NO:1, the sequence shown in SEQ ID NO:3, and both the sequence shown in SEQ ID NO:1 and SEQ ID NO:3;
 - f) a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;
 - g) a monoclonal antibody that binds to an epitope comprising residues 82-87 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;
 - h) a monoclonal antibody that binds to an epitope comprising residues 82-89 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

- i) a monoclonal antibody that competes with the monoclonal antibody CHIR-5.9 or CHIR-12.12 in a competitive binding assay;
- j) the monoclonal antibody of preceding item a) or a monoclonal antibody of any one of preceding items c)-i), wherein said antibody is recombinantly produced; and
- k) a monoclonal antibody that is an antigen-binding fragment of a monoclonal antibody of any one of preceding items a)-j), wherein said fragment retains the capability of specifically binding to said human CD40 antigen.

10 2. The method of claim 1, wherein said monoclonal antibody binds to said human CD40 antigen with an affinity (K_D) of at least about 10^{-6} M to about 10^{-12} M.

15 3. The method of claim 1, wherein said fragment is selected from the group consisting of a Fab fragment, an $F(ab')_2$ fragment, an Fv fragment, and a single-chain Fv fragment.

20 4. A method for treating a human subject for chronic lymphocytic leukemia (CLL), comprising administering to said subject an effective amount of an antagonist anti-CD40 monoclonal antibody that specifically binds Domain 2 of human CD40 antigen, wherein said antibody is free of significant agonist activity when bound to Domain 2 of human CD40 antigen.

25 5. The method of claim 4, wherein said antibody is a human antibody.

6. The method of claim 4, wherein said antibody is recombinantly produced.

30 7. The method of claim 4, wherein said antibody has the binding specificity of an antibody selected from the group consisting of the antibody produced by hybridoma cell line 5.9 and the antibody produced by hybridoma cell line 12.12.

8. The method of claim 4, wherein said antibody is selected from the group consisting of the antibody produced by the hybridoma cell line deposited with the ATCC as Patent Deposit No. PTA-5542 and the antibody produced by the hybridoma cell line deposited with the ATCC as Patent Deposit No. PTA-5543.

5

9. The method of claim 4, wherein said antibody has the binding specificity of monoclonal antibody CHIR-12.12 or CHIR-5.9.

10. The method of claim 4, wherein said antibody binds to an epitope comprising residues 82-87 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12.

11. The method of claim 4, wherein said antibody is selected from the group consisting of:

15 a) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:2, the sequence shown in SEQ ID NO:4, the sequence shown in SEQ ID NO:5, both the sequence shown in SEQ ID NO:2 and SEQ ID NO:4, and both the sequence shown in SEQ ID NO:2 and SEQ ID NO:5;

20 b) a monoclonal antibody having an amino acid sequence encoded by a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of the sequence shown in SEQ ID NO:1, the sequence shown in SEQ ID NO:3, and both the sequence shown in SEQ ID NO:1 and SEQ ID NO:3;

25 c) a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 12.12;

d) a monoclonal antibody that binds to an epitope comprising residues 82-87 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

e) a monoclonal antibody that competes with the monoclonal antibody CHIR-12.12 in a competitive binding assay;

30 f) a monoclonal antibody of any one of preceding items a)-e), wherein said antibody is recombinantly produced; and

g) a monoclonal antibody that is an antigen-binding fragment of the CHIR-12.12 monoclonal antibody or an antigen-binding fragment of a monoclonal antibody of any one of preceding items a)-f), where the fragment retains the capability of specifically binding to said human CD40 antigen.

5

12. A method for inhibiting the growth of chronic lymphocytic leukemia (CLL) cells expressing CD40 antigen, said method comprising contacting said cells with an effective amount of a human anti-CD40 monoclonal antibody that is capable of specifically binding to said CD40 antigen, said monoclonal antibody being free of significant agonist activity when bound to CD40 antigen, wherein said antibody is selected from the group consisting of:

a) the monoclonal antibody CHIR-5.9 or CHIR-12.12;
b) the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;

c) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:6, the sequence shown in SEQ ID NO:7, the sequence shown in SEQ ID NO:8, both the sequence shown in SEQ ID NO:6 and SEQ ID NO:7, and both the sequence shown in SEQ ID NO:6 and SEQ ID NO:8;

d) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:2, the sequence shown in SEQ ID NO:4, the sequence shown in SEQ ID NO:5, both the sequence shown in SEQ ID NO:2 and SEQ ID NO:4, and both the sequence shown in SEQ ID NO:2 and SEQ ID NO:5;

e) a monoclonal antibody having an amino acid sequence encoded by a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of the sequence shown in SEQ ID NO:1, the sequence shown in SEQ ID NO:3, and both the sequence shown in SEQ ID NO:1 and SEQ ID NO:3;

f) a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 5.9 or 12.12;

g) a monoclonal antibody that binds to an epitope comprising residues 82-87 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

- h) a monoclonal antibody that binds to an epitope comprising residues 82-89 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;
- i) a monoclonal antibody that competes with the monoclonal antibody CHIR-5.9 or CHIR-12.12 in a competitive binding assay;

5 j) the monoclonal antibody of preceding item a) or a monoclonal antibody of any one of preceding items c)-i), wherein said antibody is recombinantly produced; and

- k) a monoclonal antibody that is an antigen-binding fragment of a monoclonal antibody of any one of preceding items a)-j), wherein said fragment retains the capability of specifically binding to said human CD40 antigen.

10

13. The method of claim 12, wherein said monoclonal antibody binds to human CD40 antigen with an affinity (K_D) of at least about 10^{-6} M to about 10^{-12} M.

15 14. The method of claim 12, wherein said fragment is selected from the group consisting of a Fab fragment, an $F(ab')_2$ fragment, an Fv fragment, and a single-chain Fv fragment.

20 15. A method for inhibiting the growth of chronic lymphocytic leukemia (CLL) cells expressing CD40 antigen, said method comprising contacting said cells with an effective amount of an antagonist anti-CD40 monoclonal antibody that specifically binds Domain 2 of human CD40 antigen, wherein said antibody is free of significant agonist activity when bound to Domain 2 of human CD40 antigen.

25 16. The method of claim 15, wherein said antibody is a human antibody.

30 17. The method of claim 15, wherein said antibody is recombinantly produced.

35 18. The method of claim 15, wherein said antibody has the binding specificity of an antibody selected from the group consisting of the antibody produced by hybridoma cell line 5.9 and the antibody produced by hybridoma cell line 12.12.

19. The method of claim 15, wherein said antibody is selected from the group consisting of the antibody produced by the hybridoma cell line deposited with the ATCC as Patent Deposit No. PTA-5542 and the antibody produced by the
5 hybridoma cell line deposited with the ATCC as Patent Deposit No. PTA-5543.

20. The method of claim 15, wherein said antibody has the binding specificity of monoclonal antibody CHIR-12.12 or CHIR-5.9.

10 21. The method of claim 15, wherein said antibody binds to an epitope comprising residues 82-87 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12.

15 22. The method of claim 15, wherein said antibody is selected from the group consisting of:

20 a) a monoclonal antibody comprising an amino acid sequence selected from the group consisting of the sequence shown in SEQ ID NO:2, the sequence shown in SEQ ID NO:4, the sequence shown in SEQ ID NO:5, both the sequence shown in SEQ ID NO:2 and SEQ ID NO:4, and both the sequence shown in SEQ ID NO:2 and SEQ ID NO:5;

b) a monoclonal antibody having an amino acid sequence encoded by a nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of the sequence shown in SEQ ID NO:1, the sequence shown in SEQ ID NO:3, and both the sequence shown in SEQ ID NO:1 and SEQ ID NO:3;

25 c) a monoclonal antibody that binds to an epitope capable of binding the monoclonal antibody produced by the hybridoma cell line 12.12;

d) a monoclonal antibody that binds to an epitope comprising residues 82-87 of the human CD40 sequence shown in SEQ ID NO:10 or SEQ ID NO:12;

30 e) a monoclonal antibody that competes with the monoclonal antibody CHIR-12.12 in a competitive binding assay;

f) a monoclonal antibody of any one of preceding items a)-e), wherein said antibody is recombinantly produced; and

g) a monoclonal antibody that is an antigen-binding fragment of the CHIR-12.12 monoclonal antibody or an antigen-binding fragment of a monoclonal antibody of any one of preceding items a)-f), where the fragment retains the capability of specifically binding to said human CD40 antigen.

FIGURE 1A**CHIR 12.12 light chain:****leader:****MALPAQLLGLLMLWVSGSSQ****variable:****DIVMTQSPLSLTVTPGEPAISCRSSQSLLYSNGNYLDWYLQKPGQSPQLVISLGS
NRAASGVFDRFGSGSGTDFTLKISRVEAEVGVYYCMQARQTFFGPGTKVDIR****constant:****RTVAAPSVFIFPPSDEQLKRGTAQVVCLNNFVPREAKVQWKVDNALQSGNSQESVT
EQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC****FIGURE 1B****CHIR-12.12 heavy chain:****leader:****MEFGLSWVFLVAILRGVQC****variable:****QVQLVESGGVVQPGRSRLSCAASGFTFSSYGMHWVRQAPGKGLEWVAVI8YEEEN
RYHADSVKGRFTISRDNSKITLYLQMNLSRTEDTAVYYCARDGGIAAPGPDYWGQQGT
LTVVSS****constant:****ASTKGPSVFPLAPASKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAML
QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPNTKVDKRVEPKSCDKTHTCPPCPA
PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKENWYVDGVEVHNAK
TKPRREQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE
PQVYTLPPREEMTKNQVSLTCLVKGFYPSDIATEGESNGQOPENNYKTTFFVLDSDG
SFLYVSKLTVDKSRWQGNVFSCSVNHEALHNHYTQKSLSLSPGK****alternative constant region:****ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAML
QSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPNTKVDKRVEPKSCDKTHTCPPCPA
PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKENWYVDGVEVHNAK
TKPRREQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE
PQVYTLPPREEMTKNQVSLTCLVKGFYPSDIATEGESNGQOPENNYKTTFFVLDSDG
SFLYVSKLTVDKSRWQGNVFSCSVNHEALHNHYTQKSLSLSPGK**

FIGURE 2A

DNA sequence of light chain of CHIR-12.12:

FIGURE 2B

DNA sequence of heavy chain of CHIR-12.12 (including introns):

3/9

FIGURE 3A**CHIR-5.9 light chain:****leader:**

MALLAQQLGLLMLWVPGSSG

variable:AIVMTQPPPLSSPVTLGGPASISCRSSQSLVHSDGNTYLNWLQQRPGQPPRLLIYKFF
RRLSGVPDFRGSGAGTDFTLKIISRVEAEDVGVYYCMQVTQFPHTFGQGTRLEIK**constant:**RTVAAPSVFIFPPSDEQLKSGTASVVCLNNFYPREAKVQWKVDNALQSGNSQESV
EQDSKDSTYSLSSTLTKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC**FIGURE 3B****CHIR-5.9 heavy chain:****leader:**

MGSTAILALLLAVLQGVCA

variable:EVQLVQSGAEVKKPGESALKISCKGSGYFTSYWIGWVRQMPGKGLEWMGIIYPGDSD
TRYSPSFQGQVTISADKSISTAYLQWSSLKASDTAMYYCARGTAAGRYYYYYGMDV
WGQGTTTVTVBS**constant:**ASTKGPSVFPLAPASAKSTSGGTAALGCLVKDYFPEPVTVWSNSGALTSGVHTFP
QSSGLYSLSSVVTVPSSSLGTQTYICNVNHPNSNTKVDKRVEPKSCDKTHTCPPCPA
PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKEFNWYVDGVEVHN
AKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR
EQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESENQOPENNYKTPPVLDSDG
SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK**alternative constant region:**ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVWSNSGALTSGVHTFP
QSSGLYSLSSVVTVPSSSLGTQTYICNVNHPNSNTKVDKRVEPKSCDKTHTCPPCPA
PELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKEFNWYVDGVEVHN
AKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPR
EQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESENQOPENNYKTPPVLDSDG
SFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

FIGURE 4A

Coding sequence for short isoform of human CD40:

FIGURE 4B

Encoded short isoform of human CD40:

1 mivrlplqovl wgcillavhp eppiacrokq ylinsqoosl oqpgqkvlvsd cteftstecl
61 pcgesefldt wnrethchqh kycdpnlglr vqqkgktseid ticlceegwh clseacescv
121 lhrscspgfg vkqiagvsd ticcpcpvgf fsnvssafek chpwirspga aespaggdphh
181 lrdpvcvhplg aglyqkyygqe anq

FIGURE 4C

Coding sequence for long isoform of human CD40:

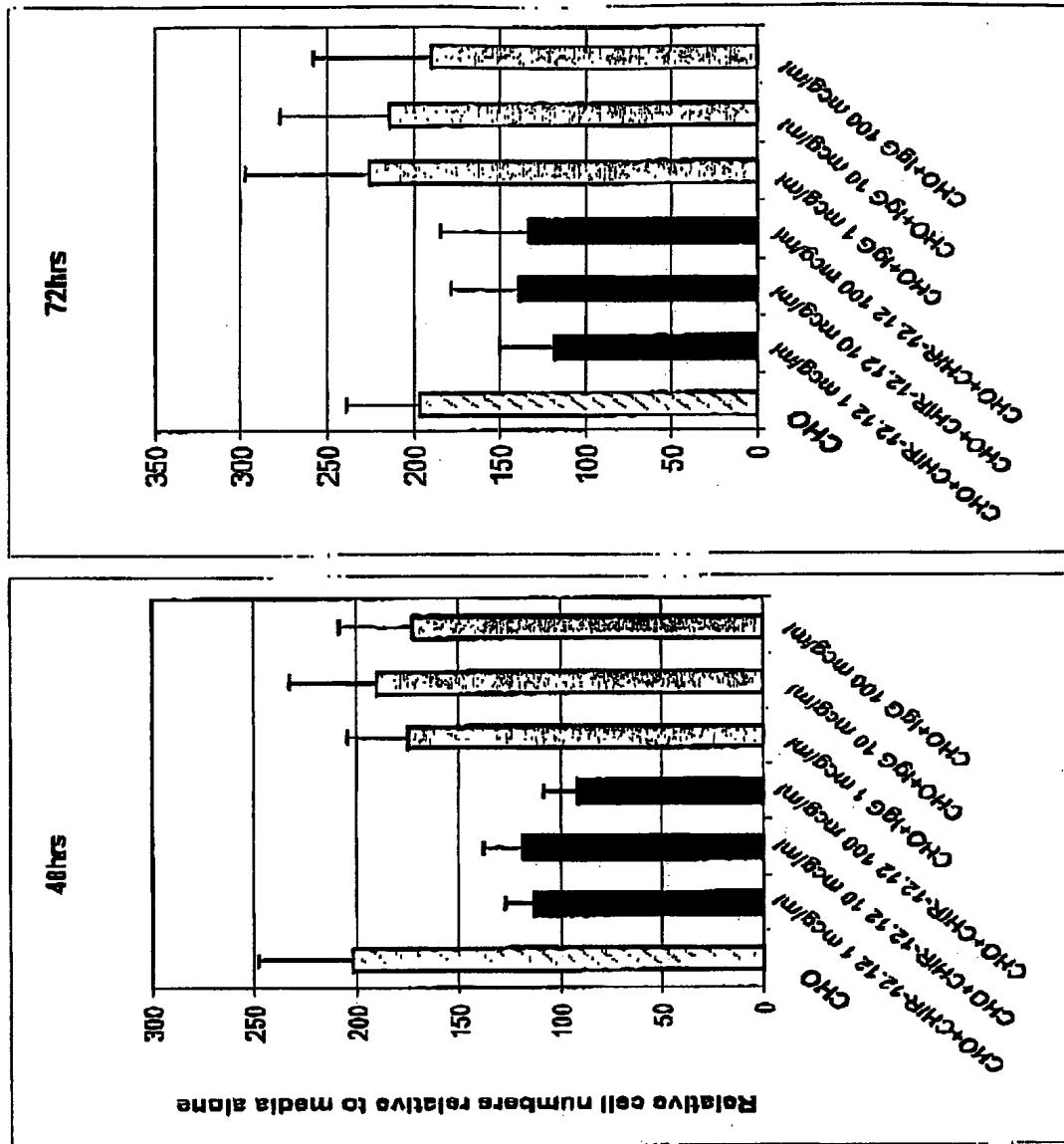
1 alggcgcgtc tgccclcgca gggcgcccc tggggcgct tgcgtaccgc tgccatcca
61 gaaccaccca ctgcgtcgag agaaaaacag tacctaataa acagtcatgt ctgttlltg
121 tgcacagcgag gacagaaact ggtgagtgac tgcacagagt taactgaaaao ygaalyccll
181 ccttcgggtg aaagcgaalt ccitagacacc tggaaatcagag agacacacly ccaccaggac
241 uuuuulgcg accccccccc tggggttogg gtcacagcaga agggcacctc agaaacacagc
301 accatcgtcga cctgtgaaga aggcgtggcac tgcacgatgg aggcctgtga gagcgttgtc
361 ctgcaccgct calgtctgtcc cggcclllyy gtcacaggcaga tgcacagg gggttctgtat
421 acoatctgcg agccctgtcgc agcgtggc ttcaccaalg tgcaalcgtt ttcgaaaaaa
481 tgcacccctt ggacaaggctg tgagacaaaa gaccggggly gtcacaggc aggcacaaac
541 aagacgtatgt tgcgtgtgg tccccaggat cggctgagag cccctgggtt gatccccalc
601 aucttggga lccgttgtc caaccccttg gtgtgtgtt ttatcaaaaa ggtggccraag
661 uaggcaaccca alaaggcccc ccaccccaag caggaacccc aggagaccaa tttccccac
721 gatcttccgt gtcaccaacat tgctgtcca gtgcaggaga ctttatcatgg atgccaacctg
781 gtcacccagg aggtatggcaa agagagtcgc alictgcgtc agggugagaoa giga

FIGURE 4D

Encoded long isoform of human CD40:

1 mvrplqlqvl wgcrltavhp epptacrekq ylinsqccsl cqpqgklvsd cleflecl
61 pcgesefldt wrnrethchqh kycdpnlglr vqqkgklseid ticiceegwh ctseacesev
121 lhrcscpgfg vkqiatgvsd ticepcpvgsf fsnvssafek chpwtsccfk dlvvqqagtu
181 lctdvvcgppqd rrlalvvipi ifgilsuill vlvfikkvak kptnkaphpk qspqeinfpd
241 dlpqgentaap vqetlhgcqp vlgqedgkesr isvqerq

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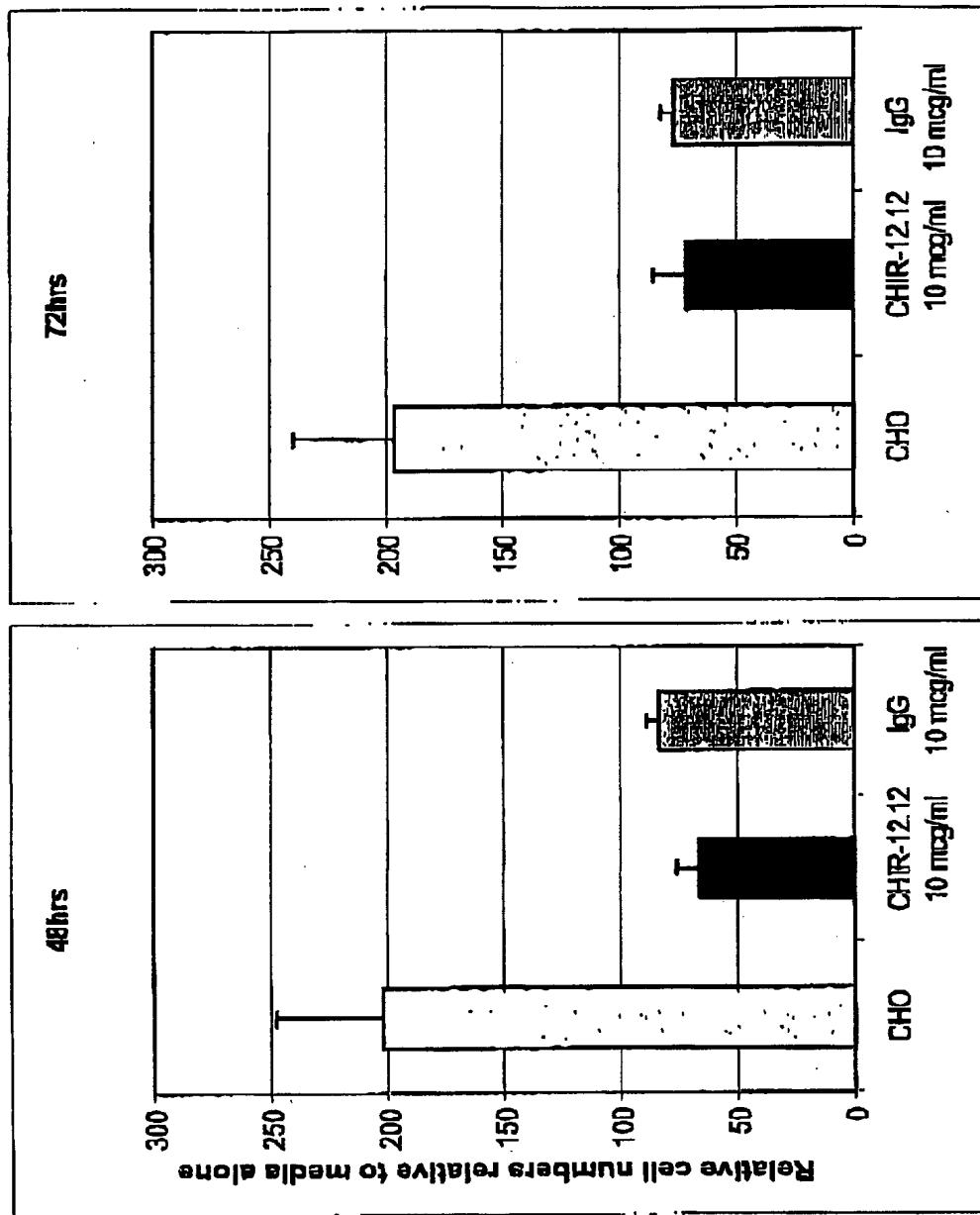


FIGURE 6B

FIGURE 6A

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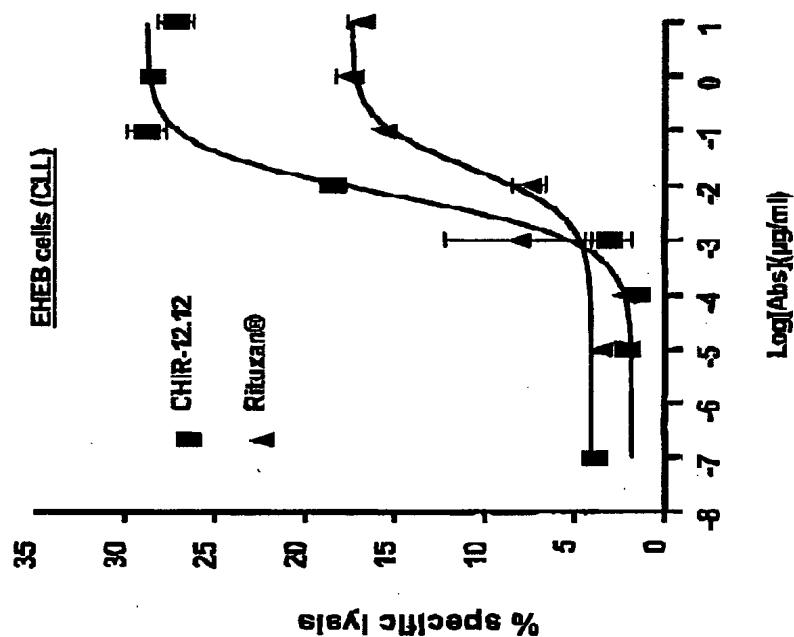
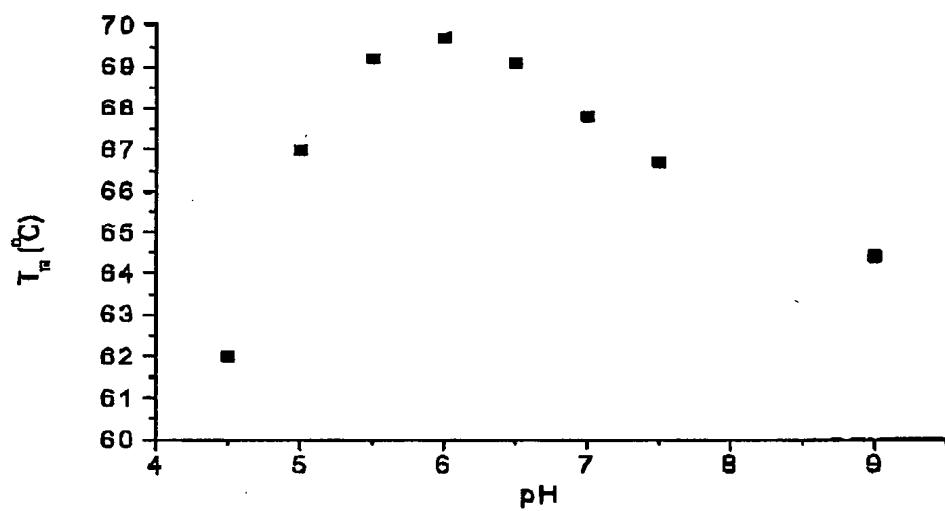


FIGURE 7

θ/θ **FIGURE 8**

SEQUENCE LISTING

<110> Aukerman, Lea
Long, Li
Luqman, Mohammad
Yabannavar, Asha
Zaror, Isabel

<120> Use of Antagonist Anti-CD40 Monoclonal Antibodies for Treatment of Chronic Lymphocytic Leukemia

<130> PP22708.002 (284267)

<150> 60/611,794
<151> 2004-09-21

<150> 60/565,710
<151> 2004-04-27

<150> 60/525,579
<151> 2003-11-26

<150> 60/517,337
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<170> FastSEQ for Windows Version 4.0

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<212> DNA
<213> Artific

<220>
<223> Coding sequence for light chain of CHIR-12.12
human anti-CD40 antibody

<221> CDS
<222> (1) . . . (720)

<400> 1
atg gcg ctc cct gct cag ctc ctg ggg ctg cta atg ctc tgg gtc tct 48
Met Ala Leu Pro Ala Gln Leu Leu Gly Leu Leu Met Leu Trp Val Ser
1 5 10 15

```

gga tcc agt ggg gat att gtg atg act cag tct cca ctc tcc ctg acc   96
Gly Ser Ser Gly Asp Ile Val Met Thr Gln Ser Pro Leu Ser Leu Thr
          20           25           30

```

gtc acc cct gga gag ccg gcc tcc atc tcc tgc agg tcc agt cag agc 144
 Val Thr Pro Gly Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser
 35 40 45

ctc ctg tat agt aat gga tac aac tat ttg gat tgg tac ctg cag aag 192
 Leu Leu Tyr Ser Asn Gly Tyr Asn Tyr Leu Asp Trp Tyr Leu Gln Lys
 50 55 60

```

cca ggg cag tct cca cag gtc ctg atc tct ttg ggt tct aat cgg gcc      240
Pro Gly Gln Ser Pro Gln Val Leu Ile Ser Leu Gly Ser Asn Arg Ala
   65           70           75           80

```

tcc ggg gtc cct gac agg ttc agt ggc agt gga tca ggc aca gat ttt 288
Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe
85 90 95

aca ctg aaa atc agc aga gtg gag gct gag gat gtt ggg gtt tat tac	336
Thr Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr	
100	105
	110
 tgc atg caa gct cga caa act cca ttc act ttc ggc cct ggg acc aaa	384
Cys Met Gln Ala Arg Gln Thr Pro Phe Thr Phe Gly Pro Gly Thr Lys	
115	120
	125
 gtg gat atc aga cga act gtg gct gca cca tct gtc ttc atc ttc ccg	432
Val Asp Ile Arg Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro	
130	135
	140
 cca tct gat gag cag ttg aaa tct gga act gcc tct gtt gtg tgc ctg	480
Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu	
145	150
	155
	160
 ctg aat aac ttc tat ccc aga gag gcc aaa gta cag tgg aag gtg gat	528
Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp	
165	170
	175
 aac gcc ctc caa tcg ggt aac tcc cag gag agt gtc aca gag cag gac	576
Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp	
180	185
	190
 agc aag gac agc acc tac agc ctc agc acc ctg acg ctg agc aaa	624
Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys	
195	200
	205
 gca gac tac gag aaa cac aaa gtc tac gcc tgc gaa gtc acc cat cag	672
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln	
210	215
	220
 ggc ctg agc tcg ccc gtc aca aag agc ttc aac agg gga gag tgt tag	720
Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys *	
225	230
	235

<210> 2
<211> 239
<212> PRT
<213> Artificial Sequence

<220>
<223> Light chain of CHIR-12.12 human anti-CD40 antibody

<400> 2	
Met Ala Leu Pro Ala Gln Leu Leu Gly Leu Leu Met Leu Trp Val Ser	
1	5
	10
	15
Gly Ser Ser Gly Asp Ile Val Met Thr Gln Ser Pro Leu Ser Leu Thr	
20	25
	30
Val Thr Pro Gly Glu Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser	
35	40
	45
Leu Leu Tyr Ser Asn Gly Tyr Asn Tyr Leu Asp Trp Tyr Leu Gln Lys	
50	55
	60
Leu Leu Tyr Ser Asn Gly Tyr Asn Tyr Leu Asp Trp Tyr Leu Gln Lys	
55	60
Pro Gly Gln Ser Pro Gln Val Leu Ile Ser Leu Gly Ser Asn Arg Ala	
65	70
	75
	80
Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe	
85	90
	95
Thr Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr	
100	105
	110
Cys Met Gln Ala Arg Gln Thr Pro Phe Thr Phe Gly Pro Gly Thr Lys	
115	120
	125
Val Asp Ile Arg Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro	
130	135
	140
Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu	

145	150	155	160
Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp			
	165	170	175
Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp			
	180	185	190
Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys			
	195	200	205
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln			
	210	215	220
Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys			
	225	230	235

```
<210> 3
<211> 2016
<212> DNA
<213> Artificial Sequence
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<220>
<223> Coding sequence for heavy chain of CHIR-12.12
human anti-CD40 antibody (with introns)

<400> 3
atggagtttggctgagctggtttccttgttgctattttaaagggtgtccagtgtcag60
gtgcagtttgtgactgggtctggggaggcgctgtccagccctggaggtccctgagactctcc120
tgtgcagcccttgattcaccttcagtagctatggcatgcaactgggtccgcagactcca180
ggcaagggcctggagttgggtggcagttataatcatatgaggaaagtaatagataccatgca240
gactccgtgaaggccgattcaccatctccagagacaatttcaagatcacgctgtatctg300
caaatacgaacgcctcagaacatgaggacacgtctgttattactgtgcgagagatgggggt360
atagcagcacctggcctgatctatgggc cagggaaaccctggtcacccgtctccatcgca420
agttacaaggccccatccggctgttgcaggacacgttcccccgttcaagagacactctggggg480
acagcggccctgggtcgctgttcaaggacacccatcccccgttcaagagacactctggggg540
aactcagggcccttgaccaggttgcaccatccggccatcccccgttcaagagacactctggggg600
ctctactccccatcagcagcgtgttgcaccatccggccatcccccgttcaagagacactctggggg660
atctgcaacgtgaatcacaaacccagcaacaccagggttcaagagagtttgcaggacactctggggg720
ccagcacaggaggggatgttgcgttgcaggacacccatcccccgttcaagagacactctggggg780
tcccggttatgttgcgttgcaggacacccatcccccgttcaagagacactctggggg840
gaggccctctgccccccatccggccatcccccgttcaagagacactctggggg900
tctgggcaggcacaggctgttgcaggacacccatcccccgttcaagagacactctggggg960
tgggctcagatctggccaaaggccatcccccgttcaagagacactctggggg1020
ccaaaggccaaactctccatcccccgttcaagagacactctggggg1080
aacttcccaatcttctctgttgcaggacacccatcccccgttcaagagacactctggggg1140
gtgcccaggttgcaggacacccatcccccgttcaagagacactctggggg1200
agtagcctgttgcaggacacccatcccccgttcaagagacactctggggg1260
cctcagcaccatcaggatgttgcaggacacccatcccccgttcaagagacactctggggg1320
acaccctcatgttgcaggacacccatcccccgttcaagagacactctggggg1380
aagaccctgatgttgcaggacacccatcccccgttcaagagacactctggggg1440
caaagcccgccgaggacacccatcccccgttcaagagacactctggggg1500
tgcaccaggttgcaggatgttgcaggacacccatcccccgttcaagagacactctggggg1560
cagcccccattgttgcaggacacccatcccccgttcaagagacactctggggg1620
cacatggacatgttgcaggacacccatcccccgttcaagagacactctggggg1680
tcttccctatgttgcaggacacccatcccccgttcaagagacactctggggg1740
gagatggacatgttgcaggacacccatcccccgttcaagagacactctggggg1800
atgcggctggatgttgcaggacacccatcccccgttcaagagacactctggggg1860
gtgttgcacttgcaggacacccatcccccgttcaagagacactctggggg1920
tggcaggcaggatgttgcaggacacccatcccccgttcaagagacactctggggg1980
acgcagaagaatgttgcaggacacccatcccccgttcaagagacactctggggg2016

<210> 4
<211> 469
<212> PRT
<213> Artificial Sequence

<220>
<223> Heavy chain of CHIR-12.12 human anti-CD40 antibody

<400> 4

Met Glu Phe Gly Leu Ser Trp Val Phe Leu Val Ala Ile Leu Arg Gly
 1 5 10 15
 Val Gln Cys Gln Val Gln Leu Val Glu Ser Gly Gly Val Val Gln
 20 25 30
 Pro Gly Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
 35 40 45
 Ser Ser Tyr Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
 50 55 60
 Glu Trp Val Ala Val Ile Ser Tyr Glu Glu Ser Asn Arg Tyr His Ala
 65 70 75 80
 Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Ile
 85 90 95
 Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Thr Glu Asp Thr Ala Val
 100 105 110
 Tyr Tyr Cys Ala Arg Asp Gly Gly Ile Ala Ala Pro Gly Pro Asp Tyr
 115 120 125
 Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly
 130 135 140
 Pro Ser Val Phe Pro Leu Ala Pro Ala Ser Lys Ser Thr Ser Gly Gly
 145 150 155 160
 Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val
 165 170 175
 Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe
 180 185 190
 Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
 195 200 205
 Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val
 210 215 220
 Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Arg Val Glu Pro Lys
 225 230 235 240
 Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu
 245 250 255
 Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr
 260 265 270
 Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val
 275 280 285
 Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val
 290 295 300
 Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser
 305 310 315 320
 Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu
 325 330 335
 Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala
 340 345 350
 Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro
 355 360 365
 Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln
 370 375 380
 Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala
 385 390 395 400
 Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr
 405 410 415
 Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu
 420 425 430
 Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser
 435 440 445
 Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser
 450 455 460
 Leu Ser Pro Gly Lys
 465

<210> 5
 <211> 469
 <212> PRT
 <213> Artificial Sequence

<220>

<223> Heavy chain of variant of CHIR-12.12 human
anti-CD40 antibody

<400> 5

Met Glu Phe Gly Leu Ser Trp Val Phe Leu Val Ala Ile Leu Arg Gly
 1 5 10 15
 Val Gln Cys Gln Val Gln Leu Val Glu Ser Gly Gly Gly Val Val Gln
 20 25 30
 Pro Gly Arg Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
 35 40 45
 Ser Ser Tyr Gly Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu
 50 55 60
 Glu Trp Val Ala Val Ile Ser Tyr Glu Glu Ser Asn Arg Tyr His Ala
 65 70 75 80
 Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ser Lys Ile
 85 90 95
 Thr Leu Tyr Leu Gln Met Asn Ser Leu Arg Thr Glu Asp Thr Ala Val
 100 105 110
 Tyr Tyr Cys Ala Arg Asp Gly Gly Ile Ala Ala Pro Gly Pro Asp Tyr
 115 120 125
 Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly
 130 135 140
 Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly
 145 150 155 160
 Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val
 165 170 175
 Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe
 180 185 190
 Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
 195 200 205
 Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val
 210 215 220
 Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys Arg Val Glu Pro Lys
 225 230 235 240
 Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu
 245 250 255
 Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr
 260 265 270
 Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val
 275 280 285
 Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val
 290 295 300
 Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser
 305 310 315 320
 Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu
 325 330 335
 Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala
 340 345 350
 Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro
 355 360 365
 Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln
 370 375 380
 Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala
 385 390 395 400
 Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr
 405 410 415
 Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu
 420 425 430
 Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser
 435 440 445
 Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser
 450 455 460
 Leu Ser Pro Gly Lys
 465

<210> 6
<211> 239
<212> PRT
<213> Artificial Sequence

<220>
<223> Light chain of CHIR-5.9 human anti-CD40 antibody

<400> 6
Met Ala Leu Leu Ala Gln Leu Leu Gly Leu Leu Met Leu Trp Val Pro
1 5 10 15
Gly Ser Ser Gly Ala Ile Val Met Thr Gln Pro Pro Leu Ser Ser Pro
20 25 30
Val Thr Leu Gly Gln Pro Ala Ser Ile Ser Cys Arg Ser Ser Gln Ser
35 40 45
Leu Val His Ser Asp Gly Asn Thr Tyr Leu Asn Trp Leu Gln Gln Arg
50 55 60
Pro Gly Gln Pro Pro Arg Leu Leu Ile Tyr Lys Phe Phe Arg Arg Leu
65 70 75 80
Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly Ala Gly Thr Asp Phe
85 90 95
Thr Leu Lys Ile Ser Arg Val Glu Ala Glu Asp Val Gly Val Tyr Tyr
100 105 110
Cys Met Gln Val Thr Gln Phe Pro His Thr Phe Gly Gln Gly Thr Arg
115 120 125
Leu Glu Ile Lys Arg Thr Val Ala Ala Pro Ser Val Phe Ile Phe Pro
130 135 140
Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr Ala Ser Val Val Cys Leu
145 150 155 160
Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp Lys Val Asp
165 170 175
Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu Ser Val Thr Glu Gln Asp
180 185 190
Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
195 200 205
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala Cys Glu Val Thr His Gln
210 215 220
Gly Leu Ser Ser Pro Val Thr Lys Ser Phe Asn Arg Gly Glu Cys
225 230 235

<210> 7
<211> 474
<212> PRT
<213> Artificial Sequence

<220>
<223> Heavy chain of CHIR-5.9 human anti-CD40 antibody

<400> 7
Met Gly Ser Thr Ala Ile Leu Ala Leu Leu Ala Val Leu Gln Gly
1 5 10 15
Val Cys Ala Glu Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys
20 25 30
Pro Gly Glu Ser Leu Lys Ile Ser Cys Lys Gly Ser Gly Tyr Ser Phe
35 40 45
Thr Ser Tyr Trp Ile Gly Trp Val Arg Gln Met Pro Gly Lys Gly Leu
50 55 60
Glu Trp Met Gly Ile Ile Tyr Pro Gly Asp Ser Asp Thr Arg Tyr Ser
65 70 75 80
Pro Ser Phe Gln Gly Gln Val Thr Ile Ser Ala Asp Lys Ser Ile Ser
85 90 95
Thr Ala Tyr Leu Gln Trp Ser Ser Leu Lys Ala Ser Asp Thr Ala Met
100 105 110
Tyr Tyr Cys Ala Arg Gly Thr Ala Ala Gly Arg Asp Tyr Tyr Tyr Tyr
115 120 125
Tyr Gly Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser

130	135	140
Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ala Ser Lys		
145	150	155
Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr		160
165	170	175
Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser		
180	185	190
Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser		
195	200	205
Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr		
210	215	220
Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys		
225	230	235
Arg Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys		240
245	250	255
Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro		
260	265	270
Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys		
275	280	285
Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp		
290	295	300
Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu		
305	310	315
Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu		320
325	330	335
His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn		
340	345	350
Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly		
355	360	365
Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu		
370	375	380
Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr		
385	390	395
Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn		400
405	410	415
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe		
420	425	430
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn		
435	440	445
Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr		
450	455	460
Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys		
465	470	

<210> 8

<211> 474

<212> PRT

<213> Artificial Sequence

<220>

<223> Heavy chain of variant CHIR-5.9 human anti-CD40 antibody

<400> 8

Met Gly Ser Thr Ala Ile Leu Ala Leu Leu Ala Val Leu Gln Gly		
1	5	10
Val Cys Ala Glu Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys		
20	25	30
Pro Gly Glu Ser Leu Lys Ile Ser Cys Lys Gly Ser Gly Tyr Ser Phe		
35	40	45
Thr Ser Tyr Trp Ile Gly Trp Val Arg Gln Met Pro Gly Lys Gly Leu		
50	55	60
Glu Trp Met Gly Ile Ile Tyr Pro Gly Asp Ser Asp Thr Arg Tyr Ser		
65	70	75
Pro Ser Phe Gln Gly Gln Val Thr Ile Ser Ala Asp Lys Ser Ile Ser		80
85	90	95

Thr Ala Tyr Leu Gln Trp Ser Ser Leu Lys Ala Ser Asp Thr Ala Met
 100 105 110
 Tyr Tyr Cys Ala Arg Gly Thr Ala Ala Gly Arg Asp Tyr Tyr Tyr
 115 120 125
 Tyr Gly Met Asp Val Trp Gly Gln Gly Thr Thr Val Thr Val Ser Ser
 130 135 140
 Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro Ser Ser Lys
 145 150 155 160
 Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr
 165 170 175
 Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala Leu Thr Ser
 180 185 190
 Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser
 195 200 205
 Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly Thr Gln Thr
 210 215 220
 Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys Val Asp Lys
 225 230 235 240
 Arg Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys
 245 250 255
 Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
 260 265 270
 Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
 275 280 285
 Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
 290 295 300
 Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
 305 310 315 320
 Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
 325 330 335
 His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
 340 345 350
 Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
 355 360 365
 Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu
 370 375 380
 Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
 385 390 395 400
 Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
 405 410 415
 Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
 420 425 430
 Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
 435 440 445
 Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
 450 455 460
 Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
 465 470

<210> 9
 <211> 612
 <212> DNA
 <213> Homo sapiens

<220>
 <221> CDS
 <222> (1)...(612)

<221> misc_feature
 <222> (0)...(0)
 <223> Coding sequence for short isoform of human CD40

<400> 9
 atg gtt cgt ctg cct ctg cag tgc gtc ctc tgg ggc tgc ttg ctg acc 48
 Met Val Arg Leu Pro Leu Gln Cys Val Leu Trp Gly Cys Leu Leu Thr
 1 5 10 15

gct gtc cat cca gaa cca ccc act gca tgc aga gaa aaa cag tac cta Ala Val His Pro Glu Pro Pro Thr Ala Cys Arg Glu Lys Gln Tyr Leu 20 25 30	96
ata aac agt cag tgc tgt tct ttg tgc cag cca gga cag aaa ctg gtg Ile Asn Ser Gln Cys Cys Ser Leu Cys Gln Pro Gly Gln Lys Leu Val 35 40 45	144
agt gac tgc aca gag ttc act gaa acg gaa tgc ctt cct tgc ggt gaa Ser Asp Cys Thr Glu Phe Thr Glu Thr Glu Cys Leu Pro Cys Gly Glu 50 55 60	192
agc gaa ttc cta gac acc tgg aac aga gag aca cac tgc cac cag cac Ser Glu Phe Leu Asp Thr Trp Asn Arg Glu Thr His Cys His Gln His 65 70 75 80	240
aaa tac tgc gac ccc aac cta ggg ctt cg ^g gtc cag cag aag ggc acc Lys Tyr Cys Asp Pro Asn Leu Gly Leu Arg Val Gln Gln Lys Gly Thr 85 90 95	288
tca gaa aca gac acc atc tgc acc tgt gaa gaa ggc tgg cac tgt acg Ser Glu Thr Asp Thr Ile Cys Thr Cys Glu Glu Gly Trp His Cys Thr 100 105 110	336
agt gag gcc tgt gag agc tgt gtc ctg cac cgc tca tgc tcg ccc ggc Ser Glu Ala Cys Glu Ser Cys Val Leu His Arg Ser Cys Ser Pro Gly 115 120 125	384
ttt ggg gtc aag cag att gct aca ggg gtt tct gat acc atc tgc gag Phe Gly Val Lys Gln Ile Ala Thr Gly Val Ser Asp Thr Ile Cys Glu 130 135 140	432
ccc tgc cca gtc ggc ttc ttc tcc aat gtg tca tct gct ttc gaa aaa Pro Cys Pro Val Gly Phe Phe Ser Asn Val Ser Ser Ala Phe Glu Lys 145 150 155 160	480
tgt cac cct tgg aca agg tcc cca gga tcg gct gag agc cct ggt ggt Cys His Pro Trp Thr Arg Ser Pro Gly Ser Ala Glu Ser Pro Gly Gly 165 170 175	528
gat ccc cat cat ctt cgg gat cct gtt tgc cat cct ctt ggt gct ggt Asp Pro His His Leu Arg Asp Pro Val Cys His Pro Leu Gly Ala Gly 180 185 190	576
ctt tat caa aaa ggt ggc caa gaa gcc aac caa taa Leu Tyr Gln Lys Gly Gly Gln Glu Ala Asn Gln * 195 200	612

<210> 10
<211> 203
<212> PRT
<213> Homo sapiens

<400> 10
Met Val Arg Leu Pro Leu Gln Cys Val Leu Trp Gly Cys Leu Leu Thr
1 5 10 15
Ala Val His Pro Glu Pro Pro Thr Ala Cys Arg Glu Lys Gln Tyr Leu
20 25 30
Ile Asn Ser Gln Cys Cys Ser Leu Cys Gln Pro Gly Gln Lys Leu Val
35 40 45
Ser Asp Cys Thr Glu Phe Thr Glu Thr Glu Cys Leu Pro Cys Gly Glu
50 55 60
Ser Glu Phe Leu Asp Thr Trp Asn Arg Glu Thr His Cys His Gln His
65 70 75 80
Lys Tyr Cys Asp Pro Asn Leu Gly Leu Arg Val Gln Gln Lys Gly Thr

85	90	95
Ser Glu Thr Asp Thr Ile Cys Thr Cys	Glu Glu Gly Trp His Cys Thr	
100	105	110
Ser Glu Ala Cys Glu Ser Cys Val Leu His Arg Ser Cys Ser Pro Gly		
115	120	125
Phe Gly Val Lys Gln Ile Ala Thr Gly Val Ser Asp Thr Ile Cys Glu		
130	135	140
Pro Cys Pro Val Gly Phe Phe Ser Asn Val Ser Ser Ala Phe Glu Lys		
145	150	155
Cys His Pro Trp Thr Arg Ser Pro Gly Ser Ala Glu Ser Pro Gly Gly		
165	170	175
Asp Pro His His Leu Arg Asp Pro Val Cys His Pro Leu Gly Ala Gly		
180	185	190
Leu Tyr Gln Lys Gly Gln Glu Ala Asn Gln		
195	200	

<210> 11
<211> 834
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (1)...(834)

<221> misc_feature
<222> (0)...(0)
<223> Coding sequence for long isoform of human CD40

<400> 11		
atg gtt cgt ctg cct ctg cag tgc gtc ctc tgg ggc tgc ttg ctg acc		48
Met Val Arg Leu Pro Leu Gln Cys Val Leu Trp Gly Cys Leu Leu Thr		
1	5	10
		15
gct gtc cat cca gaa cca ccc act gca tgc aga gaa aaa cag tac cta		96
Ala Val His Pro Glu Pro Pro Thr Ala Cys Arg Glu Lys Gln Tyr Leu		
20	25	30
ata aac agt cag tgc tgt tct ttg tgc cag cca gga cag aaa ctg gtg		144
Ile Asn Ser Gln Cys Cys Ser Leu Cys Gln Pro Gly Gln Lys Leu Val		
35	40	45
agt gac tgc aca gag ttc act gaa acg gaa tgc ctt cct tgc ggt gaa		192
Ser Asp Cys Thr Glu Phe Thr Glu Thr Glu Cys Leu Pro Cys Gly Glu		
50	55	60
agc gaa ttc cta gac acc tgg aac aga gag aca cac tgc cac cag cac		240
Ser Glu Phe Leu Asp Thr Trp Asn Arg Glu Thr His Cys His Gln His		
65	70	75
		80
aaa tac tgc gac ccc aac cta ggg ctt cgg gtc cag cag aag ggc acc		288
Lys Tyr Cys Asp Pro Asn Leu Gly Leu Arg Val Gln Gln Lys Gly Thr		
85	90	95
tca gaa aca gac acc atc tgc acc tgt gaa gaa ggc tgg cac tgt acg		336
Ser Glu Thr Asp Thr Ile Cys Thr Cys Glu Glu Gly Trp His Cys Thr		
100	105	110
agt gag gcc tgt gag agc tgt gtc ctg cac cgc tca tgc tcg ccc ggc		384
Ser Glu Ala Cys Glu Ser Cys Val Leu His Arg Ser Cys Ser Pro Gly		
115	120	125
ttt ggg gtc aag cag att gct aca ggg gtt tct gat acc atc tgc gag		432
Phe Gly Val Lys Gln Ile Ala Thr Gly Val Ser Asp Thr Ile Cys Glu		
130	135	140

ccc tgc cca gtc ggc ttc ttc tcc aat gtg tca tct gct ttc gaa aaa	480
Pro Cys Pro Val Gly Phe Phe Ser Asn Val Ser Ser Ala Phe Glu Lys	
145 150 155 160	
tgt cac cct tgg aca agc tgt gag acc aaa gac ctg gtt gtg caa cag	528
Cys His Pro Trp Thr Ser Cys Glu Thr Lys Asp Leu Val Val Gln Gln	
165 170 175	
gca ggc aca aac aag act gat gtt gtc tgt ggt ccc cag gat cgg ctg	576
Ala Gly Thr Asn Lys Thr Asp Val Val Cys Gly Pro Gln Asp Arg Leu	
180 185 190	
aga gcc ctg gtg atc ccc atc atc ttc ggg atc ctg ttt gcc atc	624
Arg Ala Leu Val Val Ile Pro Ile Ile Phe Gly Ile Leu Phe Ala Ile	
195 200 205	
ctc ttg gtg ctg gtc ttt atc aaa aag gtg gcc aag aag cca acc aat	672
Leu Leu Val Leu Val Phe Ile Lys Lys Val Ala Lys Lys Pro Thr Asn	
210 215 220	
aag gcc ccc cac ccc aag cag gaa ccc cag gag atc aat ttt ccc gac	720
Lys Ala Pro His Pro Lys Gln Glu Pro Gln Glu Ile Asn Phe Pro Asp	
225 230 235 240	
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Asp Leu Pro Gly Ser Asn Thr Ala Ala Pro Val Gln Glu Thr Leu His	
245 250 255	
gga tgc caa ccg gtc acc cag gag gat ggc aaa gag agt cgc atc tca	816
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Val Gln Glu Arg Gln *	
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Ile Asn Ser Gln Cys Cys Ser Leu Cys Gln Pro Gly Gln Lys Leu Val	
35 40 45	
Ser Asp Cys Thr Glu Phe Thr Glu Thr Glu Cys Leu Pro Cys Gly Glu	
50 55 60	
Ser Glu Phe Leu Asp Thr Trp Asn Arg Glu Thr His Cys His Gln His	
65 70 75 80	
Lys Tyr Cys Asp Pro Asn Leu Gly Leu Arg Val Gln Gln Lys Gly Thr	
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Ser Glu Thr Asp Thr Ile Cys Thr Cys Glu Glu Gly Trp His Cys Thr	
100 105 110	
Ser Glu Ala Cys Glu Ser Cys Val Leu His Arg Ser Cys Ser Pro Gly	
115 120 125	
Phe Gly Val Lys Gln Ile Ala Thr Gly Val Ser Asp Thr Ile Cys Glu	
130 135 140	
Pro Cys Pro Val Gly Phe Phe Ser Asn Val Ser Ser Ala Phe Glu Lys	
145 150 155 160	
Cys His Pro Trp Thr Ser Cys Glu Thr Lys Asp Leu Val Val Gln Gln	
165 170 175	
Ala Gly Thr Asn Lys Thr Asp Val Val Cys Gly Pro Gln Asp Arg Leu	
180 185 190	

Arg Ala Leu Val Val Ile Pro Ile Ile Phe Gly Ile Leu Phe Ala Ile
195 200 205
Leu Leu Val Leu Val Phe Ile Lys Lys Val Ala Lys Lys Pro Thr Asn
210 215 220
Lys Ala Pro His Pro Lys Gln Glu Pro Gln Glu Ile Asn Phe Pro Asp
225 230 235 240
Asp Leu Pro Gly Ser Asn Thr Ala Ala Pro Val Gln Glu Thr Leu His
245 250 255
Gly Cys Gln Pro Val Thr Gln Glu Asp Gly Lys Glu Ser Arg Ile Ser
260 265 270
Val Gln Glu Arg Gln
275